Mechanical or Biological Cues?
Parameters to Engineer a 3D Human Masseter Muscle

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Acknowledgements

I, Mariea Brady, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis. Signed: Date:

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Abstract

Skeletal muscle lends itself to the central tenet of tissue engineering in that myoblasts isolated from skeletal muscle tissue are able to recapitulate the exact processes that occur *in vitro* during muscle development (Shah *et al.*, 2005). *In vitro* skeletal muscle engineering involves the culture of isolated primary myogenic cells in an environment conducive to the formation of a three-dimensional (3D) tissue construct, containing differentiated myotubes, capable of generating force. The aim of this thesis was to test the effect of mechanical and biological cues on the development of a tissue engineered craniofacial masseter muscle construct. For the first time primary myogenic, non myogenic and heterogenous (mixture of myogenic and non-myogenic) cells were tested in a 3D collagen scaffold to define parameters for in vitro engineering of human masseter muscle.

The cytomechanical properties of the human masseter muscle derived cells (MDCs) in 3D were quantified over 24 hours using a culture force monitor (CFM) and the results showed a synergistic response, the heterogenous mixture generated significantly ($p<0.01$) more force than the sum of the individual components of separated cells. Further, muscle specific (myogenin, myosin heavy chain (MYHC)) and matrix remodeling (MMP-2) gene expressions were upregulated relative to the separated individual cell types.

To study mechanical (cell-matrix) cues MDCs were subject to external loading and increased matrix stiffness. The results showed no significant change in gene expression. To study biological (cell-cell) cues, MDCs were cultured at high densities and for an extended period of time (6 days). The results showed an upregulation of

muscle specific (myogenin, MYHC-IIX/D, MYHC-β) gene expression. However, MMP-2 gene expression remained unchanged. These results indicated that cell-cell signals are more important than cell-matrix signals in 3D myogenic cell differentiation. This study highlights the importance of non-myogenic cells in force generation and the effect of increasing substrate stiffness and cell density in control of construct maturation. These are the first parameters defined towards successful 3D human muscle engineering.

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Introduction

Skeletal Muscle

Skeletal muscle is the most abundant type of muscle in the body (Williams *et al.*, 1989) and is responsible for control of voluntary movement and maintenance of structural contours of the body (Riboldi *et al.*, 2005). The human body contains more than 300 muscles that differ widely in size, shape, strength, and resistance to fatigue (Pedrosa–Domello *et al.*, 2000). In the craniofacial area, skeletal muscle allows for facial expression and tongue, eye and jaw mobility (Alsberg *et al.*, 2001).

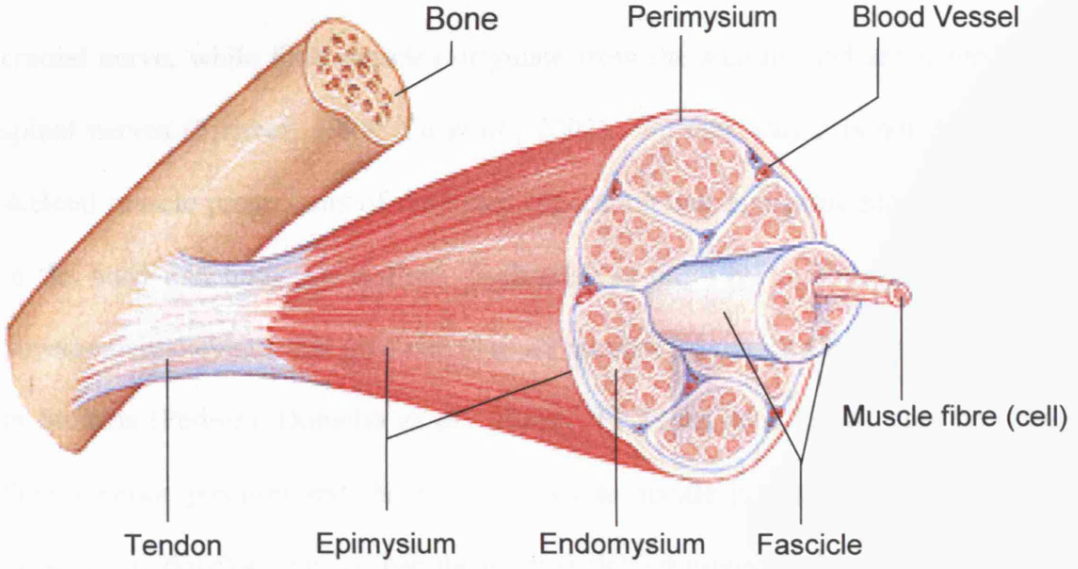

Figure 1: Skeletal muscle is a bundle within a bundle structure. Each muscle fibre is wrapped with a delicate connective tissue sheath, the endomysium. The perimysium is a collagenic sheath which surrounds bundles (or fascicles) of myofibres. The entire muscle is strengthened by a coarse epimysium sheath.

Blood vessels penetrate through the connective tissue to provide the muscle with essential nutrients (Adapted from Marieb, 1998).

Each skeletal muscle is a discrete organ composed of bundles of muscle fibres (Marieb, 1998; Figure 1). Muscle fibres are the cellular units of skeletal muscle, they are long multinucleated cells usually rounded or polygonal in cross-section varying from a diameter of 10-100μm in mature muscle (Lewis *et al.*, 2001).

In mammals the muscles of the head have a different embryological origin to the limb musculature (Goldspink, 1998). Jaw-closing muscles originate from the mesoderm of the first branchial arch and are innervated by the mandibular division of the fifth cranial nerve, while limb muscles originate from the somites and are innervated by spinal nerves (Sperber, 1989; Yu *et al.*, 2003). Further, *Pax-3* is not detectable in skeletal muscle progenitors of the head, suggesting that myogenic regulatory circuits in the head and body are distinct (Tajbakhsh *et al.*, 1997). Knowledge about the developmental events that give rise to more specialized muscles is scarce, particularly in humans (Pedrosa–Domello *et al.*, 2000). However, it is known that because of their anterior position and the infant's need to suckle just after birth, craniofacial musculature develops earlier than the other skeletal muscles (Goldspink, 1998).

Muscles of the craniofacial region are structurally and functionally more specialized than the limb muscles (Pedrosa–Domello *et al.*, 2000). Jaw muscles are active during a large variety of motor tasks, like mastication, biting, speech, and swallowing. To execute this variety of tasks, they must be able to control the position of the mandible

precisely, and instantaneously apply changing forces to it (Goldspink, 1998; Korfage *et al.*, 2005a).

The masseter is one of the mandibular elevator muscles and can be divided into a superficial, intermediate and deep portion (Soussi-Yanicostas *et al.*, 1990; Figure 2). The masseter muscle arises from the zygomatic arch (cheek bone). It has three heads which are fused together on the arch. The superficial part of the muscle is the largest. It arises from the anterior two-thirds of the lower border of the zygomatic process of the maxilla. Its fibres slope downwards and backwards and are inserted into a wide area; from the angle of the mandible (jaw bone) forwards along the lower border, and upwards to include the lower part of the ramus (McMinn, 1994).

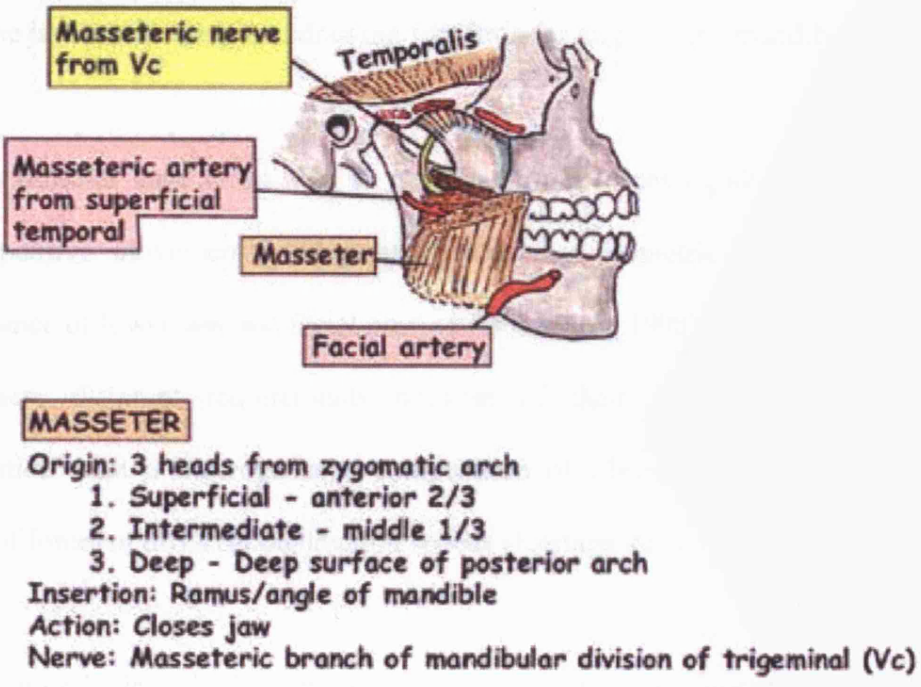

Figure 2: The masseter muscle has three heads which originate on the zygomatic arch. The superficial part of the muscle is the largest, followed by the intermediate, then deep. The muscle fibres insert along the mandible and ramus.

The masseter is innervated by the masseteric branch of the anterior division of the mandibular nerve. Its blood supply is via a branch of the superficial temporal facial artery (Source: http://www.instantanatomy.net/head neck/areas/parotidmasseter.html).

The intermediate part of the muscle arises from the middle third of the arch and the deep part from the deep surface of the arch in continuity. The fibres pass vertically downwards to be inserted into the ramus of the mandible. Their insertions fuse with each other and with the superficial fibres at the anterior border of the ramus, but are separate posteriorly. The masseter is supplied by the masseteric branch of the anterior division of the mandibular nerve. Its blood supply is via a branch of the superficial temporal or tranverse facial artery (McMinn, 1994). The masseter muscle closes the jaw by elevating and drawing forwards the angle of the mandible.

The jaw muscles have to be able to produce strong forces rapidly during incision, slow repetitive movements during mastication and isometric contraction for the maintenance of lower jaw and facial posture (Goldspink, 1998). The jaw muscles can meet these different requirements because of their complex architecture, in combination with a heterogeneous composition of fibres capable of producing a variety of forces at different contraction speeds (Korfage *et al.*, 2005a).

Skeletal muscle is a highly ordered structure from gross anatomy down to the molecular level (Stål, 1994). At the gross anatomy level, each muscle fibre is surrounded by connective tissue, and this is termed the endomysium. Bundles of endomysia are in turn surrounded by connective tissue termed the perimysium, which

in turn are encapsulated by the epimysium. The connective tissue surrounding the fibres allows for movement between fibres and transmits force to the tendon.

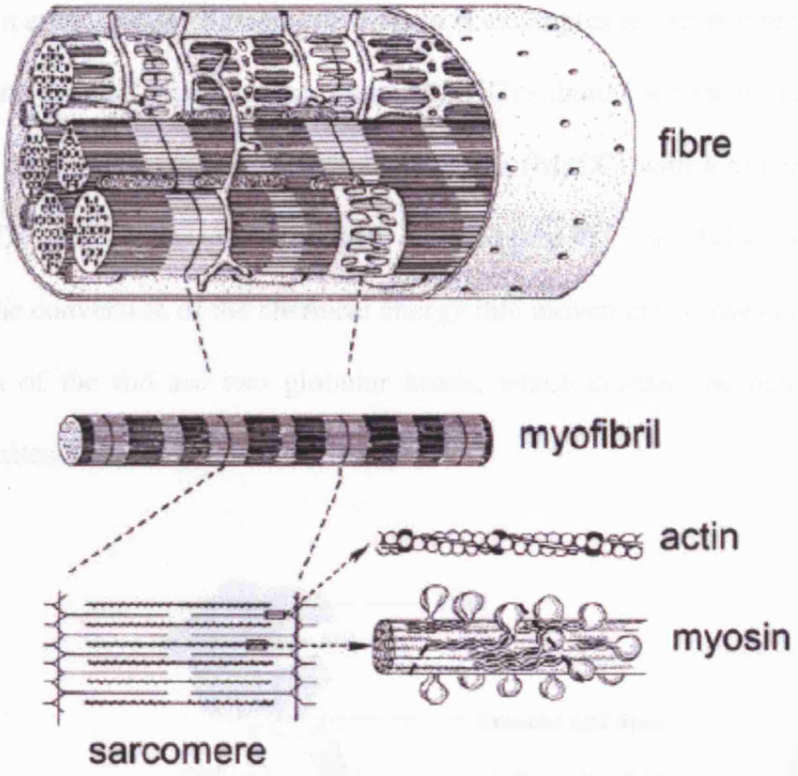

Figure 3: Skeletal muscle is highly organised at the molecular level. This diagram shows the level of order from a single fibre to a myofibril to a sarcomere and then individual myofilaments (myosin and actin) (Adapted from Korfage *et al.*, 2005a).

As well as being rich in connective tissue, skeletal muscles are highly vascularised to provide essential nutrients for muscle function (Chargé and Rudnicki, 2004).

At the molecular level order continues and muscle fibres are composed of myofibrils, which consist of aligned sarcomeres (Figure 3). A single sarcomere is the smallest contractile unit of a muscle fibre (about 2μm long) and is in turn divisible into thick and thin myofilaments, myosin and actin respectively.

Myosin is the dominating contractile protein in muscle fibres, and it is the molecular motor that converts free energy derived from the hydrolysis of ATP into mechanical work (Yu *et al.*, 2003). Sarcomeric myosin is a complex hexameric protein consisting of two anti-parallel myosin heavy chain (MyHC) subunits with a molecular weight of 200-220 kDa each and four myosin light chains (MyLC) with a molecular weight of 17-23 kDa each (Weiss and Leinwand, 1996; Figure 4). The MyLC molecules play a part in the conversion of the chemical energy into movement (Lowey *et al.*, 1993). At the ends of the rod are two globular heads, which contain the mATPase and the binding sites for actin (Stål *et al.*, 1994).

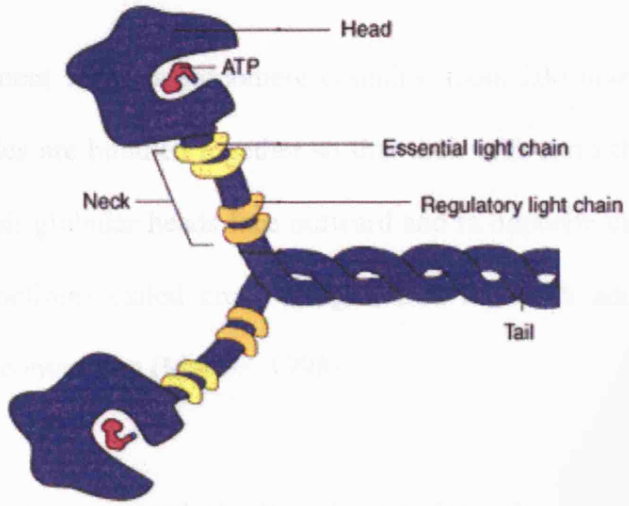

Figure 4: The sarcomeric myosin molecule is composed of 4 light-chain (MyLC) molecules (2 essential (yellow) and 2 regulatory (orange)) and 2 myosin heavy-chain (MyHC (blue)) subunits (Modified from http://fig.cox.miami.edu/~cmallery/150/neuro/myosin.jpg).

Desmin is a type III intermediate filament protein characteristic of muscle cells. Desmin filaments appear to form a structural lattice that provides support for the

contractile machinery (Lazarides, 1980). They play an important part in maintaining the relative positions of myofibrils, and also lying parallel to them in a manner suggesting that they may participate in transducing contractile forces longitudinally through the muscle cell (Tippet, 1997).

Skeletal Muscle Contraction

Huxley HE and Hanson (1954) and Huxley AE (not related) and Niedergerke (1954) published back to back papers in *Nature* independently demonstrating that muscle shortens by relative sliding between two sets of subcellular filaments containing the proteins myosin and actin. This formed the basis of 'the sliding filament theory' which is now well accepted as the mechanism for muscle contraction.

Each thick filament within a sarcomere contains about 200 myosin molecules. The myosin molecules are bundled together so that their tails form the central part of the filament and their globular heads face outward and in opposite directions at each end. The heads, sometimes called cross bridges, link the thick and thin myofilaments together during contraction (Marieb, 1998).

The thin filaments composed chiefly of actin, bear the active sites to which the myosin cross bridges attach during contraction. Several regulatory proteins are also present in the thin filament. Two strands of tropomyosin a rod shaped protein spiral about the actin core and help to stiffen it and successive tropomyosin molecules are arranged end to end along the actin filament. In a relaxed muscle fibre they block actins active sites so that the myosin heads cannot bind to the thin filaments (Marieb, 1998).

The other major protein in the thin filament is troponin, a three-polypeptide complex. One of these polypeptides is an inhibitory subunit that binds to actin. Another binds to tropomyosin and helps to position it on actin. The third polypeptide binds calcium ions (Marieb, 1998).

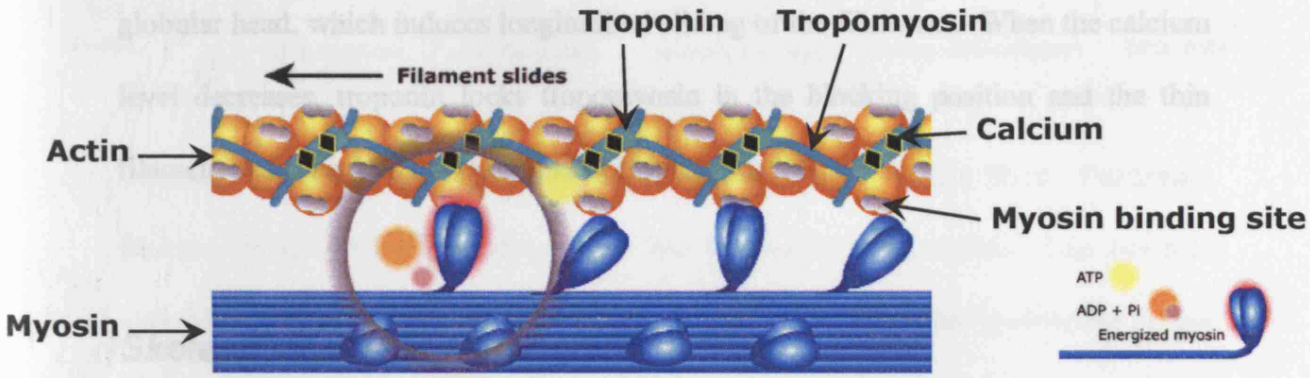

Figure 5: The sliding filament theory states that in the presence of Ca^{2+} myosin binding sites on the thin actin filament are exposed. Energised myosin binds to actin and the hydrolysis of ATP causes a power-stroke of the myosin head resulting in the thin filament sliding towards the centre of the sarcomere. The combined sliding of all the thin filaments within the muscle fibre results in contraction of the cell (Adapted from Matthews, 2001).

In the absence of calcium ions, tropomyosin blocks access to the myosin binding site of actin (Ganong, 1989). Initiation of contraction in skeletal muscle begins with a nerve impulse generating an action potential in the muscle fibres. The action potential causes releases of calcium ions which then bind to troponin. When calcium binds to troponin, the positions of troponin and tropomyosin are altered on the thin filament and myosin then has access to its binding site on actin (Matthews, 2001; Figure 5).

Myosin hydrolyses adenosine triphosphate (ATP) and undergoes a conformational change into a high-energy state. The head group of myosin binds to actin forming a cross-bridge between the thick and thin filaments. The energy stored by myosin is released, and adenosine diphosphate (ADP) and inorganic phosphate (P_i) dissociate from myosin. The resulting relaxation of the myosin molecule entails rotation of the globular head, which induces longitudinal sliding of the filaments. When the calcium level decreases, troponin locks tropomyosin in the blocking position and the thin filament slides back to the resting state (Matthews, 2001).

Skeletal Muscle Development

Most of our current knowledge about skeletal muscle development is based on studies of limb muscles, which originate from the somites and are innervated by spinal nerves (Pedrosa–Domello *et al.*, 2000). The first skeletal muscle to differentiate in the embryo is the myotome, a sheet of differentiated skeletal muscle cells that lies between the dermomyotome and the sclerotome (Maroto *et al.*, 1997).

Signals from the neural tube, notochord and paraxial mesoderm are involved in regulating somatic myogenesis (Rong *et al.*, 1992; Buffinger and Stockdale, 1994; Cossu *et al.*, 1995). The paracrine factors, Wnts and Sonic hedgehogs (Shh), in combination instruct the myotome cells to become myogenic by inducing them to synthesize key transcription factors; *Pax-3, Myf5* and *MyoD* (Maroto *et al.*, 1997; Tajbakhsh *et al.*, 1997).

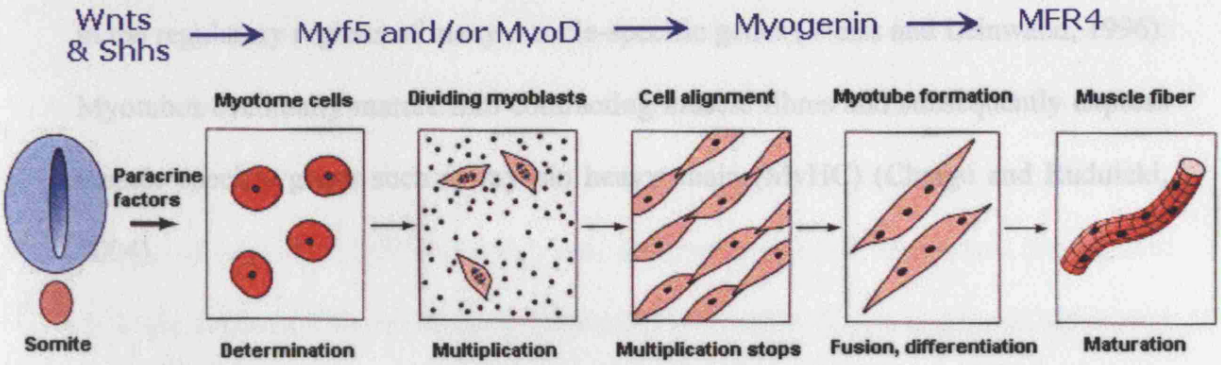

Figure 6: Skeletal muscle development from somite to muscle fibre. Paracrine factors (Wnts and Shhs) determine the myotome cells, which then become committed myoblasts (expressing Myf5 and/or MyoD) which proliferate in the presence of growth factors. When the myoblasts cease dividing, they align and fuse into myotubes (expressing myogenin). The myotubes become organized into muscle fibers (expressing MRF4) that spontaneously contract (Adapted from Gilbert, 2006).

Proliferative *Myf5* and/or *MyoD* positive myogenic cells are termed myoblasts. Myoblasts withdraw from the cell cycle to become terminally differentiated myocytes, which fuse to form myotubes, expressing the "late" myogenic regulatory factors, *myogenin* and *MRF4* (Chargé and Rudnicki, 2004; Figure 6).

The myogenic regulatory factors (MRFs) belong to subfamily A of the basic helix loop helix (bHLH) transcriptional regulators (Atchely *et al.*, 1997). The MRFs include myf5, MyoD, myogenin, and MRF4 they are considered master muscle regulatory proteins because of their ability to induce skeletal myogenesis. These proteins function as heterodimeric transcriptional activators that bind a sequence

motif termed an E-box that contains a CANNTG (N, any nucleotide) consensus found in the regulatory regions of many muscle-specific genes (Weiss and Leinwand, 1996). Myotubes eventually mature into contracting muscle fibres and subsequently express muscle specific genes such as myosin heavy chain (MyHC) (Chargé and Rudnicki, 2004).

The primary pathways involved in myoblast differentiation *in vitro* (2D) and *in vivo* have been characterized (Pedrotty *et al.*, 2005). *In vitro*, skeletal muscle precursor cells express MyoD or Myf-5 in the committed state and turn on transcription of myogenin when induced to differentiate (Hollenberg *et al.*, 1993; Ohkawa *et al.*, 2006). Chargé and Rudnicki (2004) reported that myoblast terminal differentiation is characterised by the upregulation of the MRFs myogenin and MRF4.

In summary, during embryonic development, skeletal muscle precursor cells called myoblasts exit the proliferative cycle, initiate biochemical differentiation, and fuse to form multinucleated myotubes. One of the major proteins expressed during the differentiation of myoblasts and myotubes is sarcomeric MyHC (Weiss and Leinwand, 1996; Allen and Leinwand, 2001). Myogenin and MyHC are markers of differentiated skeletal muscle (Tajbakhsh *et al.*, 1997). In the thesis to follow, the gene expression of myogenin and MyHC were monitored to mark myoblast terminal differentiation.

Myosin Heavy Chain Isoforms

Myosin heavy chain is the main component of the thick filament of the sarcomere. The myosin heavy chain is both a structural protein and an enzyme (Sciote and Morris, 2000). It enzymatically hydrolyses ATP and is therefore the key factor in

determining the nature of excitation-contraction coupling and moving (Barany, 1967). There are several isoforms, i.e. proteins which have the same function but are encoded by a different gene, of the MyHC molecule. The different isoforms of MyHC are functionally unique and cannot be substituted for one another (Allen *et al.*, 2000; Allen and Leinwand, 2001). Further they are transcriptionally competent and capable of being expressed simultaneously (Horton *et al.*, 2001). There is general agreement that the heavy chains determine the force-velocity characteristics of skeletal muscle fibres (Pedrosa–Domello *et al.*, 2000, Sciote and Morris, 2000).

The sarcomeric MyHC isoform genes found in humans include α-cardiac, β/I, IIA, IIX, IIB, extraocular (expressed in the extrinsic eye and some pharyngeal muscles), and 2 developmental forms, namely, neonatal (also named foetal, perinatal, or developmental) and embryonic (Sartore *et al.*, 1987; Butler-Browne *et al.*, 1988; Smerdu *et al.*, 1994; Stål *et al.*, 1994; Weiss and Leinwand, 1996; Sciote and Morris, 2000; Horton *et al.*, 2001). Embryonic and neonatal MyHCs are typically expressed during muscle development. MyHC- β/I is the slow contracting isoform and the three 'type II' MyHCs are fast contracting isoforms (Sciote and Morris, 2000). Another fast contracting MyHC isoform is α-cardiac primarily expressed in cardiac muscle; it also has rare tissue-specific expression in skeletal muscle, being identified in the human masseter (Bredman *et al.*, 1991; Stål, 1994; Sciote *et al.*, 1994).

The main difference between the MyHC isoforms is their rate of converting ATP into energy, which determines the speed of actin-myosin detachment. Bottinelli *et al.* (1996) established a good correlation between the MyHC isoforms and the maximum contraction velocity. At present the reported comparable shortening speeds of adult

skeletal MyHCs is extra-ocular > IIB > IIX > IIA > α-cardiac > I (Sciote and Morris, 2000). The functional differences among the various MyHC isoforms contribute to or result in changes in the physiology of the muscle in which they are expressed (Weiss and Leinwand, 1996).

Each different MyHC gene displays a pattern of expression that is tissue specific and developmentally regulated (Butler-Browne *et al.*, 1988). In the masseter MyHC gene expression is quite unusual. In addition to the typical MyHC isoforms found in healthy adult muscle (type I/β and type II), the masseter also expresses neonatal, embryonic and/or α–cardiac myosin heavy chains (Sciote *et al.*, 1994). Further, individual fibres in the masseter co-expressed up to four different MyHC isoforms (Stål *et al.*, 1994). Yu *et al.* (2003) later reported that up to five different MyHC isoforms could be observed in the same masseter muscle fibre. A unique myofibrillar protein isoform expression has been observed in the human masseter muscle fibers, suggesting significant differences in structural and functional properties between muscle fibres from human masseter and limb muscles (Yu *et al.*, 2003).

Skeletal Muscle Fibre Types

Skeletal muscle cell fibres have been systematically classified into types (Sciote and Morris, 2000). According to the major MyHC found in adult skeletal muscle the following pure fibre types exist (see Table 1): slow type I with MyHC-I/β, and two fast types, namely IIA with MyHC-IIA and IIX with MyHC-IIX (Pette and Staron, 2000). Skeletal muscle fibres are distinguished by different contractile velocities, metabolic properties, and structural protein compositions, including varying MyHC isoform profiles (Talmadge and Roy, 1993).

Classifications:			
Myosin based classification	Slow 1/β	Fast IIA	Fast IIX
Classification based on *metabolism*	Slow Oxidative	Fast Oxidative	Fast glycolytic
Classification based on *fatigue resistance*	Slow fatigue resistant	Fast fatigue resistant	Fast fatigable
Properties:			
Myosin isoforms	MyHC-1/β	MyHC-IIA	MyHC-IIX
ATPase activity	Low	High	Very High
Resistance to fatigue	High	Intermediate	Low
Metabolism	Oxidative	Oxidative glycolytic	Glycolytic

Table 1: Classification of human skeletal muscle fibres and properties of each fibre type (adapted from Polla *et al.*, 2004)

Pure muscle fibres express only one MyHC isoform, fibres which express more than one MyHC isoform are called hybrid fibres (for example, type I + IIX, or type IIA + IIX). Compared to other skeletal muscles, cranial muscles have a wider repertoire of contractile protein expression and function (Sciote *et al.*, 2003). Studies have shown that the fibre composition of the human masseter muscle differs from that of limb/trunk muscles and has certain characteristics that can be linked to its activity (Ringqvist, 1971; 1974; Eriksson, 1982; Eriksson and Thornell, 1983; Thornell et al., 1984; Butler-Browne et al., 1988; Soussi-Yanicostas *et al.*, 1990; Bredman et al., 1991; Tuxen et al., 1992; Sciote et al., 1994; Stål et al., 1994; Monemi et al., 1996; Korfage and Van Eijden, 1999, 2003; Korfage *et al.*, 2005).

A large range of contractile properties exists among hybrid fibres containing a particular combination of MyHC isoforms. The shortening velocity of hybrid fibers is said to lie between the individual MyHC isoforms they express (Pette & Staron, 1997; Larsson and Moss, 1993; Bottinelli *et al.*, 1996). The existence of different MyHCs in a fibre allows a smooth transition of force regulation needed in movements of the jaw muscles (Butler-Browne *et al*, 1988).

The masseter muscle contains a significant proportion of hybrid fibres, termed IM and IIC which co-express slow and a "fast B like" MyHC in the masseter (Stål, 1994). However, the precise MyHC content of these fibre types is not yet fully clear (Korfage and Van Eijden, 1999). Stål (1994) reported that up to eight fibre types could be distinguished in the masseter on the basis of their pattern of expression of MyHC isoforms.

Histochemical staining of healthy adult limb muscle has demonstrated that skeletal muscle is composed of a mosaic of type I and II fibres, with the type II fibres being of larger diameter (Sciote and Morris, 2000). Human jaw-closing muscles are predominantly composed of type I fibres (60-70%) in the adult (Soussi-Yanicostas *et al.*, 1990) and the type II fibres are much smaller in diameter than limb muscle fibres (Ringqvist, 1971; Eriksson, 1982; Eriksson and Thornell, 1983).

There have been several attempts to explain the variety of hybrid muscle fibres of the masseter muscle;

1. **Transition:** hybrid fibres are thought to arise when there is an alteration from one type into another type of muscle fibre (Korfage and Van Eijden, 1999). They can change their phenotype in response to several influences by switching different isoform genes on or off (Goldspink, 1998). During transition, the fibres will change from a pure fiber type, into another pure fiber type, via hybrid fibre types that express the old as well as the new MyHC isoforms. A suggestion was made of the following transition pathway of MyHC isoforms: Iβ ↔ Iβ/IIA ↔ IIA ↔ IIA/IIX ↔ IIX (Gorza, 1990; DeNardi *et al.* 1993). It might therefore be concluded that the jaw muscles are continuously switching from one fiber type to another (Korfage *et al.*, 2005b).

2. **Innervation:** another possible explanation for the many hybrid fibers in the jaw muscles could be due to a difference in innervation as compared with limb and trunk muscles, i.e., branchial *vs.* spinal (Stål *et al.*, 1987).

3. **Development:** the many hybrid fibers in adult jaw muscles could also be regarded as a characteristic of development. During development fibres can change their phenotype, during which hybrid fibres arise (Butler-Browne *et al.*, 1988).

4. **Regeneration:** during the regeneration process, fibers transiently start to express MyHC-foetal and MyHC-I (Yang *et al.*, 1997). The continued expression of these fibre types in adult jaw muscles might indicate a longer time period for jaw muscles to heal. The masseter is known to heal badly after an injury (Pavlath *et al.*, 1998).

5. **Allotype:** alternatively, hybrid fibers can be seen as a legitimate stable phenotype, or an allotype, as named by Hoh (1991), and not as a transitional phenotype (Stephenson, 1999; Lutz and Lieber, 2000). A fibre integrates several of the stimuli it receives (like neural activity, hormones, stretch, etc.) and produces a response to them that signals the expression of MyHC gene transcription (Korfage *et al.*, 2005b).

Skeletal Muscle Regeneration

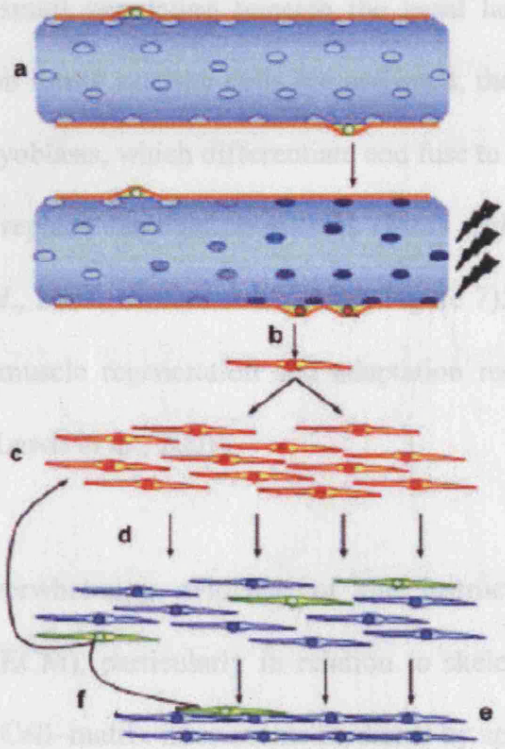

Figure 7: Model of satellite cell dynamics (adapted from Collins *et al.*, 2005). (a) Quiescent satellite cells reside beneath the basal lamina (orange) of a myofiber. Satellite cells are activated after injury (b). Daughter cells migrate from beneath the basal lamina and the satellite cell progeny proliferate extensively (c). The progeny adapt alternative fates and either commit to differentiation (blue) or

remain undifferentiated (yellow) (d). Cells committed to differentiation fuse to repair of replace damaged myofibers (e). Undifferentiated progenitors continue proliferating to derive the next cohort of myoblasts or are captured beneath the basal lamina of newly formed myofibers and renew the satellite cell population (f).

Skeletal muscle has a remarkable ability to regenerate following damage (Chargé and Rudnicki, 2004). The capacity to regenerate resides in a population of mononucleated satellite cells (Beauchamp *et al.*, 2000). The satellite cell is a quiescent muscle precursor forming a small population beneath the basal lamina of each myofiber (Mauro, 1961). Upon injury satellite cells are activated, the muscle precursor cells then form a pool of myoblasts, which differentiate and fuse to provide the myo-nuclei required to repair or replace damaged myofibers (Moss and Leblond, 1971; Snow, 1978; Boudreault *et al.*, 2001; Collins *et al.*, 2005; Figure 7). The cellular processes seen during skeletal muscle regeneration and adaptation recapitulate those evident during development (Lewis *et al.*, 2001).

Further, there is overwhelming evidence of the instructional capacity of the extracellular matrix (ECM), particularly in relation to skeletal muscle regeneration (Lewis *et al.*, 2001). Cell–matrix interactions mediated by specific cell receptors and cell binding epitopes on many matrix molecules not only play a dominant role in cell attachment and migration, but also regulate or promote cellular differentiation and gene expression levels (Gelse *et al*, 2003).

Skeletal Muscle ECM Remodeling

The primary function of the ECM is to endow tissues with their specific mechanical and biochemical properties. For many years, the ECM was thought to be created by connective tissue cells and surround those cells as a mere structural scaffold (Velleman, 1998). However, this is now regarded as an oversimplification as resident cells are responsible for the synthesis and maintenance of the ECM, which in turn, has an impact on cellular functions (Gelse *et al*, 2003).

ECM molecules are thought to serve as more than a mechanical link of ECM/integrin/cytoskeleton in the transduction of forces into and out of cells. Physical distortion of the ECM results in cytoskeletal remodeling and signals alterations in intracellular biochemistry such as cell proliferation. The inverse also holds: changes in cytoskeletal tension results in remodeling of structural scaffolds within the ECM and within neighboring cells, thereby altering biochemistry outside the cell (Ingber, 2002; Pattison *et al.*, 2003).

The connective tissue component of skeletal muscle plays an important role in maintaining the structure of the muscle and also in providing an environment in which the contractile muscle fibres can function (Grounds et al., 1998). *In vivo*, individual muscle fibres are elongated and are embedded in a protein- and carbohydrate-rich ECM. The ECM of skeletal muscles needs to adapt continually to changing environmental demands. Changes in masticatory muscle structure and function may be developmental, as seen in anomalies of facial form, or adaptive as a result of surgical prodecures such as orthognathic surgery or after use of orthodontic appliances (Carmeli *et al.*, 2004).

The remodeling of connective tissue is an integral feature of normal growth and development (Reynolds *et al.*, 1994; Reynolds and Meikle, 1997). The mechanisms underlying remodeling of intramuscular connective tissue are complex and multifactorial and involve ECM molecules (collagenous glycoproteins, non-collagenous glycoproteins, proteoglycans and elastin), receptors for the ECM (integrins) and enzymes that remodel the ECM (matrix metalloproteinases) (Lewis *et al.*, 2001). Further, tissue remodeling is usually tightly regulated by the interplay of cell-cell and cell-matrix interactions involving the production of enzymes, activators, inhibitors and regulatory molecules such as cytokines and growth factors (Reynolds and Meikle, 1997).

The matrix metalloproteinases (MMPs) are a family of zinc-dependent matrix-degrading enzymes that appear to be obligatory for the maintenance of tissue ECM homeostasis (Matrisian, 1990; Woessner, 1991; Corcoran *et al.*, 1996). In the last decade, MMPs have been shown to have a role in the maintenance of the extracellular matrix in a wide range of normal and pathological tissues, although relatively little is known regarding their role in muscle (Carmeli *et al.*, 2004). In normal biology, these enzymes are involved in a great number of cellular processes including motility, migration and invasion (Lewis *et al.*, 2001). Regulating the activity of the MMPs at both mRNA and/or protein levels modulates the degradation of the ECM components (Corcoran *et al.*, 1996). The MMPs can be inhibited by a special class of specific tissue inhibitors of metalloproteinases (TIMPS) (Matrisian, 1992). The activity of MMPs on ECM substrate is dependent on the balance between the enzymes and their inhibitors (Lewis *et al.*, 2001).

One of the most important MMPs associated with skeletal muscle function and dysfunction appears to be MMP2, also known as gelatinase A, or 72kDa type IV collagenase. MMP2, by regulating the integrity and composition of the ECM in skeletal muscle, plays essential roles in myofiber proliferation, differentiation, fiber healing after injury, and maintenance of surrounding connective tissue (Matrisian, 1992; Carmeli *et al.*, 2004). Investigations have shown the expression of MMP-2 activity and mRNA in all phases of the differentiation of myoblasts to myotubes (Lewis *et al.*, 2001).

Craniofacial tissue loss

Craniofacial tissue loss due to congenital defects, disease, and traumatic injury is a major clinical problem (Alsberg *et al.*, 2001; Bach *et al.*, 2004). These and other complex craniofacial problems can have a profound physiological and psychological effect; therefore, many techniques have been developed to effectively and aesthetically rebuild the head and face including methods that require both scaffolding and competent cell populations for effective tissue regeneration (Warren *et al.*, 2003; Shah *et al.*, 2005).

Contemporary dental practice is largely based on conventional, non-cell-based therapies that rely on durable materials from outside the patient's body. Amalgam, composites, metallic implants, synthetic materials, and tissue grafts from human cadavers and other species have been the mainstream choices for the restoration of dental, oral, and craniofacial structures (Mao *et al.*, 2006). Despite various levels of clinical success, conventional therapies possess inherent limitations, such as donor site morbidity, an obligatory graft resorption phase, disease transmission, major histo-

incompatibility, graft-versus-host disease, immunosuppression, structural failure, stress shielding, and infection of foreign material (Warren *et al.*, 2003).

The current technique to replace missing craniofacial skeletal muscle is the surgical transfer of local or free flaps (Shah *et al.*, 2005). Muscle transfers are needed to rehabilitate the paralyzed mouth, re-animate the eye and restore masticatory function (Tardy and Kastenbauer, 1995). However, surgical harvesting of a significant mass of muscle causes functional loss and volume deficiency and increases the susceptibility to infection at the donor site, moreover, the transferred tissue typically is not designed to function in its new capacity and may degenerate prior to integration into its new site (Bach *et al.*, 2003a). The lack of availability of functional substitutes of skeletal muscle hampers the reconstruction of this native tissue (Beier *et al.*, 2006). Therefore, there is a significant craniofacial clinical need for skeletal muscle that has only begun to be addressed by the tissue engineering field (Alsberg *et al.*, 2001).

Tissue Engineering

Recently Williams (2006) defined tissue engineering as "the creation of new tissue for the therapeutic reconstruction of the human body, by the deliberate and controlled stimulation of selected target cells, through a systematic combination of molecular and mechanical signals". The field of tissue engineering, through the application of engineering and biological principles, has the potential to create functional replacements for damaged or pathologic tissues (Alsberg *et al.*, 2001). Specifically, craniofacial tissue engineering promises the regeneration or *de novo* formation of dental, oral, and craniofacial structures (Mao *et al.*, 2006). To date cells have been seeded onto natural (e.g., collagen, alginate, agarose, hyaluronic acid derivatives, chitosan, and fibrin glue), synthetic (e.g., polyglycolide (PGA), polylactides (PLLA,

PDLA), polycaprolactone (PCL), and polydioxanone (PDS)), and mineral (polycrystalline ceramic (hydroxyl apatite)) scaffolds (Warren *et al.*, 2003).

To date three main approaches to tissue engineering have been pursued conduction, induction, and cell transplantation (Langer and Vacanti, 1993; Putnam and Mooney, 1996; Figure 8). In the conduction approach a naturally derived or synthetic matrix acts as a passive three-dimensional mechanical scaffold on which cells can attach, proliferate, migrate, and differentiate (Alsberg *et al.*, 2001).

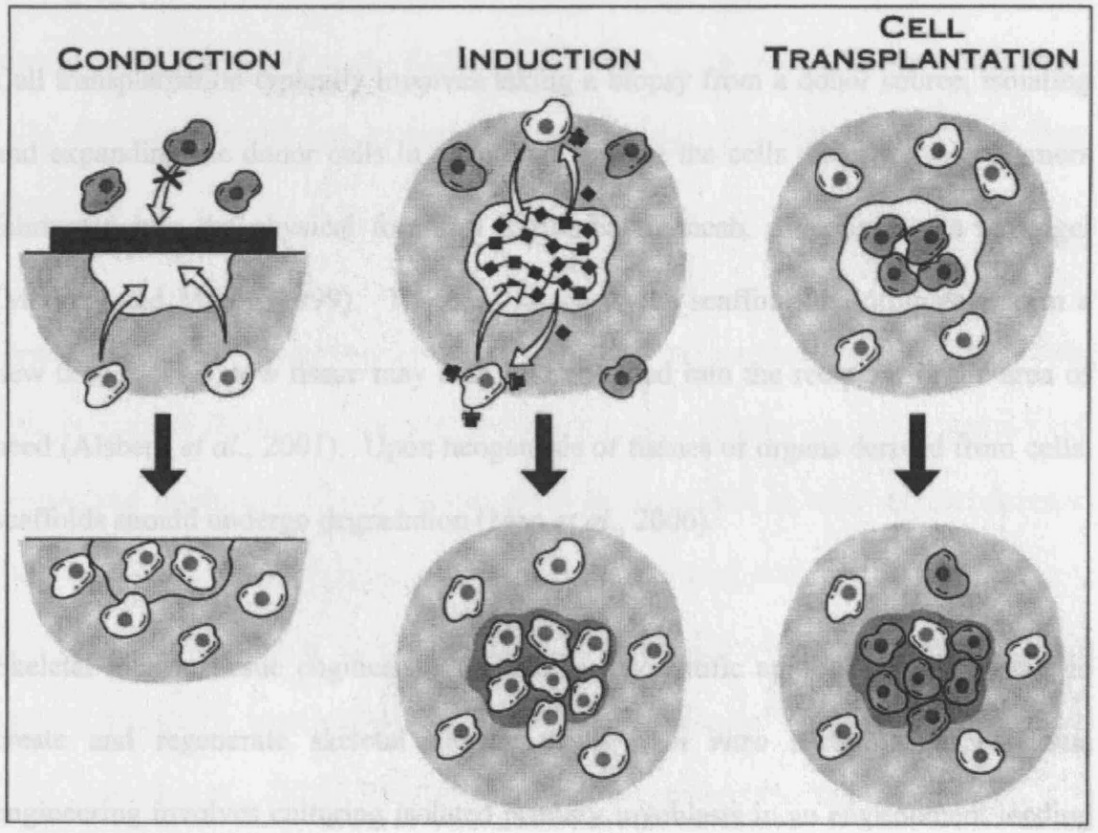

Figure 8: Three approaches to tissue engineering: conduction, in which a scaffold acts as a barrier controlling which cells can infiltrate the area; induction, in which cells are induced to migrate to desired location by controlling the surrounding matrix or cell-cell interactions; and cell transplantation, where

donor cells are seeded directly onto a scaffold *in vitro* and the cell-scaffold construct can be implanted to a defect site (Source: Alsberg *et al.*, 2001).

The induction approach is based on the premise that adherent cells receive signals from the surrounding cell-matrix and these signals can be controlled (Alsberg *et al.*, 2001). Some induction approaches from the literature include: attaching specific peptides or proteins to a scaffold (Peppas and Langer, 1994; Tamura et al., 1997), controlling the mechanical properties of the scaffold (Opas, 1989; Karamichos *et al.*, 2006) and manipulating cell-cell interactions (Putnam and Mooney, 1996).

Cell transplantation typically involves taking a biopsy from a donor source, isolating and expanding the donor cells in vitro, and seeding the cells directly onto polymers fabricated into the physical forms of a fiberbased mesh, a sponge, or a hydrogel (Mooney and Mikos, 1999). The cells attach to the scaffold and ultimately form a new tissue. This new tissue may then be implanted into the recipient in the area of need (Alsberg *et al.*, 2001). Upon neogenesis of tissues or organs derived from cells, scaffolds should undergo degradation (Mao *et al.*, 2006).

Skeletal muscle tissue engineering represents a scientific approach that attempts to create and regenerate skeletal muscle tissue. *In vitro* skeletal muscle tissue engineering involves culturing isolated primary myoblasts in an environment leading to the formation of a three-dimensional differentiated muscle tissue construct. Success depends on the regenerative properties of the satellite cells and their potential for proliferation and differentiation. Differentiation is the fusion of single muscle cells to form multinucleated myotubes. It is necessary to investigate novel

approaches for culturing functional, differentiated 3D skeletal muscle constructs *in vitro* using primary myoblasts. Further, an understanding of the molecular control mechanisms of muscle development, differentiation and maturation is important.

Skeletal Muscle Constructs

To date there have been a number of attempts to tissue engineer skeletal muscle *in vitro* (Vandenburgh, 1983 onwards; Dennis and Kosnik, 2000; Dennis *et al.*, 2001; Kosnik *et al.*, 2001; Okano *et al.*, 1997; Mulder *et al.*, 1998; Okano and Matsuda, 1998a; 1998b; Acartuk *et al.*, 1999; Saxena *et al.*, 1999, 2001; Powell *et al.*, 1999; 2002; Cheema *et al.*, 2003; 2004; Bach *et al.*, 2003; 2004; 2006; Riboldi *et al.*, 2005; Huang *et al.*, 2005; Shah *et al.*, 2005). However, progress has been slow and to date a fully functional tissue engineered skeletal muscle construct has not yet been developed.

Vandenburgh (1983) pioneered skeletal muscle tissue engineering and reported the first 3D model of skeletal muscle, providing a 3D environment for myogenic cell growth and application of mechanical strain to the muscle culture. It is understood that complex mechanical forces generated in the growing embryo play a crucial role in organogenesis. During embryogenesis, the continuous passive tension applied to skeletal muscle by bone growth directs the initial phases of organogenesis (Stewart, 1972; Powell *et al.*, 2002). Therefore subsequent groups have used mechanical cues to tissue engineer skeletal muscle. Okano *et al.* (1997) and Cheema *et al.* (2003) used a 3D type I collagen gel seeded with a high density of C2C12 myoblasts and mechanically stretched these constructs to produce tissue engineered muscle. Okano *et al.* (1997) used a process of cellular packing through centrifugation, which helped increase the cell density in the collagen gels (Okano *et al.* 1998 (a)). Cyclic stretching

was employed which resulted in a highly orientated hybrid muscular tissue in which both the cells and the collagen fibres were aligned in the direction of stretch (Okano *et al.* 1998 (a)). In addition to this, a capillary network was introduced to the muscular tissues by implanting the *in vitro* muscular tissue grafts into nude mice (Okano *et al.* 1998 (b)). Cheema *et al.* (2003) seeded C2C12s at a high cell density (20 million/5ml collagen gel) to create a differentiated skeletal muscle construct. Mechanical stimulation of the 3D collagen constructs containing fused C2C12 myoblasts resulted in up-regulation of IGF-I gene splicing and production, and it is likely that changes in myofibre size may be affected through this mechanism (Cheema *et al.*, 2004).

Dennis *et al.* (2000; 2001) have engineered 3D skeletal muscle tissue 'myooids' from different primary muscle cultures. A myooid is a three-dimensional skeletal muscle construct cultured from mammalian myoblasts and fibroblasts (Kosnik *et al.*, 2001). Their sources of primary myoblasts included adult mouse, neonatal mouse and adult rat. Alongside these primary cultures, co-cultures of mouse cell line myoblasts (C2C12) and fibroblasts were used (Dennis *et al.* 2000, 2001). The C2C12-fibroblast co-cultures contracted singly and sporadically, whereas myooids produced from primary cells contracted regularly and vigorously as a syncytium (Dennis *et al.* 2001). Further, C2C12-fibroblast myooids generated the least amount of force when excited by transverse electrical stimulation. Co-cultures of the myoblast and the fibroblast cell lines indicated that fibroblasts provide important cues, promoting myooid formation (Dennis *et al.* 2001). The major limitations of these tissue engineered cultures was the lack of further progression of myotubes to express adult phenotype in terms of sarcomere structure, excitability and contractility (Vandenburgh *et al.*, 1989; 1990; 1991).

Powell *et al.* (1999; 2002) reported the tissue engineering of human bioartificial muscles (HBAMs) by suspending muscle cells (derived from the human vastus lateralis) in collagen/MATRIGEL, casting in a silicone mold containing end attachment sites, and allowing the cells to differentiate. The resulting HBAMs were representative of skeletal muscle in that they contained parallel arrays of postmitotic myofibers; however, they differ in many other morphological characteristics (Powell *et al.*, 1999). However, they fall short of actual skeletal muscle in many aspects, including small diameter muscle fibers, low myofiber density, and excessive ECM. To be used for structural/functional skeletal muscle repair or replacement, HBAMs need to be engineered with properties more similar to in vivo skeletal muscle (Powell *et al.*, 2002).

For *in vitro* skeletal muscle tissue engineering to become a reality, cells need to interact with a scaffold material that can provide physical support particularly in the early stages of tissue formation until the tissue is able to supports its own growth and development by the production of growth factors and extracellular matrix components (Shah *et al.*, 2005). Many researchers have focused attention on exploring and developing biomaterial scaffolds for skeletal muscle constructs; including elastomeric materials and salinised glass. Acartuk *et al.* (1999) examined the initial attachment, morphological characteristics, and proliferative behaviour of murine C2C12 myoblasts on glass substrata to control myotube alignment for tissue engineering skeletal muscle reporting the use of glass as a viable biomaterial scaffold. Similarly, Shah *et al.* (2005) used a 3D phosphate glass fibre construct to tissue engineer craniofacial muscle demonstrating that phosphate-based glass fibres could support the *in vitro* attachment, proliferation and differentiation of primary human muscle derived cells.

To determine the utility of elastomeric materials as substrates for tissue engineering skeletal muscle constructs Mulder *et* al. (1998) cultured G8 skeletal myoblasts on commercially available polyurethane Tecoflex®. The myoblasts attached, proliferated, displayed migratory activity and differentiated into multinucleated myotubes which expressed myosin heavy chain, indicative that the myotubes had reached a state of terminal differentiation. The results of the study suggested that it was indeed feasible to engineer bioartificial muscle constructs consisting of skeletal myoblasts cultivated on a 3-D elastomeric substrate (Mulder *et al.*, 1998). Riboldi *et al.* (2005) similarly investigated an elastomeric stubstrate as a scaffold for skeletal muscle tissue engineering applications, testing the suitability of a known biodegradable block copolymer (DegraPol®) processed by electrospinning in the novel form of microfibrous membranes, as scaffold for skeletal muscle tissue engineering. Cell viability, adhesion and differentiation on coated and uncoated DegraPol® slides were investigated using the cell lines, C2C12 (mouse myogenic cell line) and L6 (rat myogenic cell line)) and primary human satellite cells (HSCs). Positive staining for myosin heavy chain expression indicated that differentiation of C2C12 multinucleated cells had occurred. Suggesting that the results of their study provided significant evidence of the suitability of electrospun DegraPol® membranes as scaffolds for skeletal muscle constructs (Riboldi *et al.*, 2005).

Clearly, many attempts have been made so far to construct skeletal muscle tissue *in vitro*, together the results of these studies suggest that it is indeed feasible to engineer bioartificial muscles; nonetheless, with current technology, tissue-engineered skeletal muscle analogues are far from being a clinical reality (Riboldi *et al.*, 2005). These 3D

engineered muscle constructs can be used to determine the effects of specific stimuli on both the functional (force production, endurance capacity, and contractile dynamics) and molecular (level and isoform of contractile and regulatory proteins) development of skeletal muscle (Huang *et al.*, 2005) as well as future applications in therapeutic replacement of tissues lost to damage due to disease and/or trauma (Lewis *et al.*, 2008).

Thesis Overview

Although progress has been made in the construction of muscle organoids tissue engineering of skeletal muscle still remains a major challenge (Bach *et al.*, 2006). The purpose of this study was to develop an *in vitro* craniofacial skeletal muscle construct using primary human muscle derived cells from the masseter seeded within a 3D type I collagen gel scaffold. This study investigated the effect of mechanical and biological cues as the myogenic cells were induced to differentiate within the construct.

Materials and Methods

1. Primary Cell Culture

As part of on-going research at the Eastman Dental Hospital and Institute, masseter muscle samples were previously obtained from patients undergoing the following surgical procedures under general anaesthesia:

- Bimaxillary orthognathic surgery
- Mandibular orthognothic surgery
- Removal of mandibular third molars

The removal of biopsy tissue was approved by, and in accordance with, the guidelines of the Human Subjects Ethics Committee. Samples were only taken from healthy adult patients and informed consent was obtained prior to all biopsies.

Biopsies were taken from the deep surface of the anterior border of the superficial belly of the masseter muscle via an intra-oral incision at the time of surgery (Boyd *et al.*, 1984). Biopsies were washed with antibiotic (penicillin, 1000u/ml; streptomycin, 100μg/ml; fungizone, 2.5 μg/ml; Invitrogen, Scotland, UK)-supplemented Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Scotland, UK), scissor-minced and any visible pieces of connective tissue were removed in order to prevent fibroblast overgrowth.

The tissue fragments were plated into 0.2% gelatin coated flasks with a surface area of 80 cm^2, incubated in standard growth medium (20% fetal calf serum (FCS, First Link Ltd, Birmingham, UK), 1% penicillin (1000u/ml)/streptomycin(100µg/ml) (P/S;

Gibco Chemicals, Paisley, UK) in DMEM) and maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. The standard growth medium used in this study has high levels of serum, which are typically used to allow myogenic cells to proliferate in culture (Yaffe, 1971). The first wave of migration of mononuclear cells from the explant was designated the 'α-wave', and this population was used throughout this study (Lewis *et al.*, 2000).

The expanded cells were stored under cryogenic conditions. The cryogenic storage of cells is the ability to store small aliquots of frozen cells in liquid nitrogen (-196°C) without loss of proliferative or differentiative capacity. This allows replication of experiments and comparative studies (Blau and Webster, 1981). Freezing is facilitated by dimethylsulphoxide (DMSO), which is a cryopreservative that reduces the formation of ice crystals in the cells. The cells used in this study were retrieved from cryogenic storage.

Gelatin coating of flasks and coverslips

All anchorage dependent cells need to attach to a solid substrate in order to proliferate. Tissue culture flasks are made from polystyrene, which is manufactured to produce a negative surface charge in order to aid cell adhesion, but this surface will not support myoblast proliferation (Freshney, 1992). Myoblasts adhere more efficiently when there is a net positive charge, therefore all tissue flasks and cover-slips were coated with gelatin. A 0.2% working solution of gelatin was made up from a stock 2% gelatin solution (Sigma, Poole, UK) diluted with phosphate buffered saline (PBS). All flasks and coverslips were coated under sterile conditions with sufficient substrate (10mls per flask and 0.5ml per coverslip) and left for 30 minutes to allow substrate attachment. Excess substrate was poured off and flasks were ready for use.

Subculture

As primary cell cultures become established the cells become densely populated and thus closely associated with one another (confluent). Cells were closely monitored using light microscopy and when 70-80% confluency was reached subculturing was carried out. Subculture allows expansion of the culture, the possibility of cloning and characterization, but leads to the loss of any cells that have already differentiated.

Routine subculture was as follows: growth medium was removed from the culture vessels and discarded. All reagents were warmed at 37°C. The cell layer was washed twice with 10mls of PBS. Washing the cell layer with PBS removed most of the serum proteins and diluted those that were left, thus minimizing the inhibitory effects the proteins would have on trypsin activity.

The cells were then incubated in 7mls of 0.05% trypsin in 0.53mM EDTA (Gibco, Paisley, UK) for 5 minutes. Trypsin digests extracellular matrix links, and EDTA was used as a chelating agent to sequester calcium and magnesium. During this period the cells become detached from the substrate. The action of trypsin was inhibited by addition of 10mls of serum containing growth medium. The uniform cells suspension was then centrifuged at 2000rpm for 5 minutes and the supernatant discarded.

The cell pellet was disrupted by firmly tapping the tube several times and the cells were resuspended in 10mls of standard growth medium. A 1:3 split was achieved by adding an equal amount of the cell suspension to each of the three gelatin coated flasks prepared. The first subculture was defined as passage one, denoted P_1, with ensuing subcultures denoted P_2, P_3 etc. The cell number was determined using a

haemocytometer and the trypan blue exclusion method. 20µl of cell suspension was placed in a sterile eppendorf tube and 20µl of trypan blue dye added in a ratio of 1:1. The trypan blue dye was taken up by the dead cells and these cells were not counted.

2. The Culture Force Monitor

The Culture Force Monitor (CFM) is a force monitoring device which enables the pattern of forces generated by cells embedded in a 3D collagen matrix to be quantified and correlated with cell motility/traction, contraction and fibril remodeling (Eastwood *et al.*, 1994; Brown *et al.*, 1998; Karamichos, 2006). The CFM monitors cell-matrix interactions as a real time force contraction profile (force plotted against time). This instrument enables the characterisation of the cytomechanical properties of different cell types within a 3D collagen construct. The CFM was designed and developed by Dr. Mark Eastwood (Eastwood *et al.*, 1994).

Collagen construct preparation

Collagen gels were cast between 2 floatation bars, which were in turn attached to stainless steel wire A-frames (Figure 9). For collagen construct preparation, 0.7 ml of 10× minimum essential medium (MEM) Eagles solution (Invitrogen, Scotland, UK) was added to 6ml of rat-tail type I collagen (First Link, Birmingham, UK) (in 0.1M acetic acid, protein concentration = 2.035 mg/g), then neutralized by titration with 5M and 1M NaOH, until a colour change (yellow to cirrus pink) was observed (Prajapati *et al.*, 2000). 1ml of this gel was then used to pre-coat 2 polyethylene mesh floatation bars. 4.5mls was added to 5×10^6 cells suspended in 0.5mls DMEM giving a 5ml gel which was poured into a $75\times25\times15mm^3$ polyethylene mould, containing the pre-coated mesh floatation bars attached to A-frames, and set within 30 minutes in an incubator maintained at 37°C in a humidified atmosphere of 5% CO_2. The gel was

then physically detached from the base of the mould and floated in 20mls of standard growth medium.

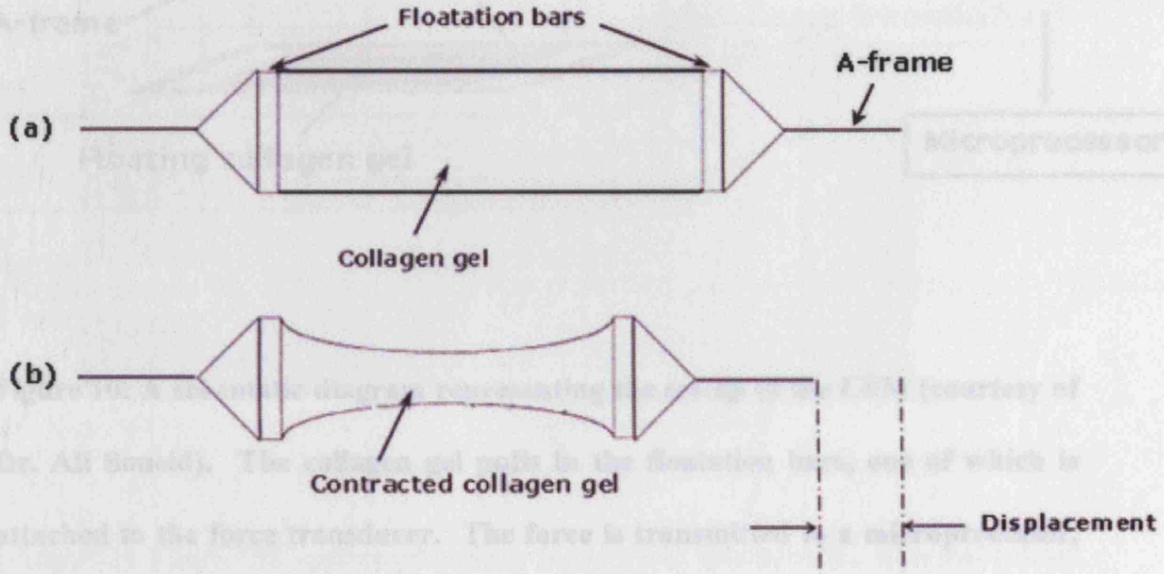

Figure 9: A schematic diagram showing the contraction of the cell seeded collagen construct. (a) The collagen gel was set between two floatation bars in a rectangular shaped well. The floatation bars were in turn connected to A-frames which facilitated attachment to the CFM. (b) As the collagen gel contracted (over 24 hours) the bars were pulled inwards and the displacement was measured.

The cell-seeded collagen construct was then tethered to the CFM and cell mediated matrix contraction was quantified (Figure 10). The ability of the CFM to measure very early force generation with great accuracy, reproducibility and with little background noise makes it ideal for investigating the mechanisms by which force is generated by cells in this system.

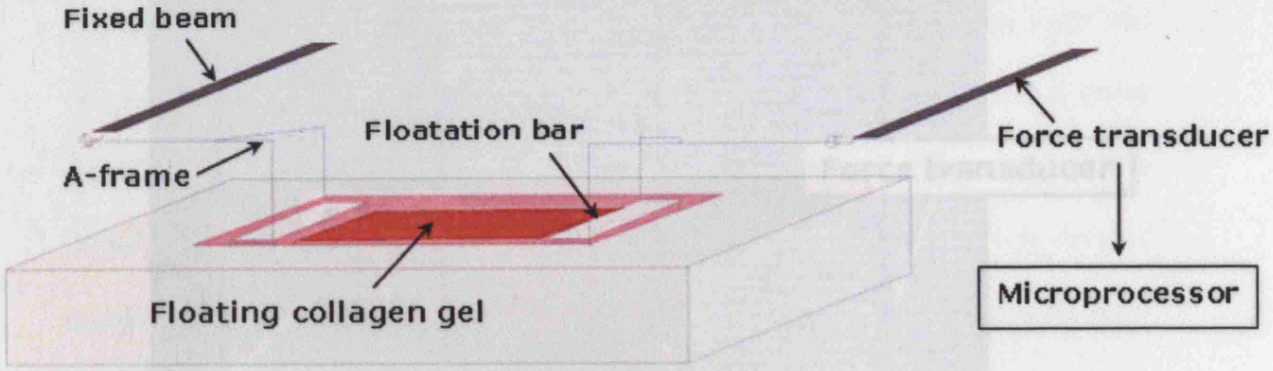

Figure 10: A schematic diagram representing the set-up of the CFM (courtesy of Dr. Ali Soueid). The collagen gel pulls in the floatation bars, one of which is attached to the force transducer. The force is transmitted to a microprocessor, the signal is amplified and converted for analysis using LabView software. Contraction profiles are plotted over 24 hours.

The CFM consists of highly sensitive force transducers (movements as small as 1μm are detectable) that are connected to a personal computer (PC) via an analogue-to-digital converter (ADC). Displacement was converted into a force reading and stored on the PC. This apparatus provides a continuous and precise measure of force generated by living cells in a 3-D collagen matrix (Eastwood *et al.*, 1998). Further, the phases of contraction from the cells over 24 hours represent a reproducible framework for interpretation of the cell behaviour (Eastwood *et al.*, 1996). The CFM was utilised in this study to monitor the force contraction profiles of craniofacial muscle derived cells embedded in collagen constructs (Figure 11).

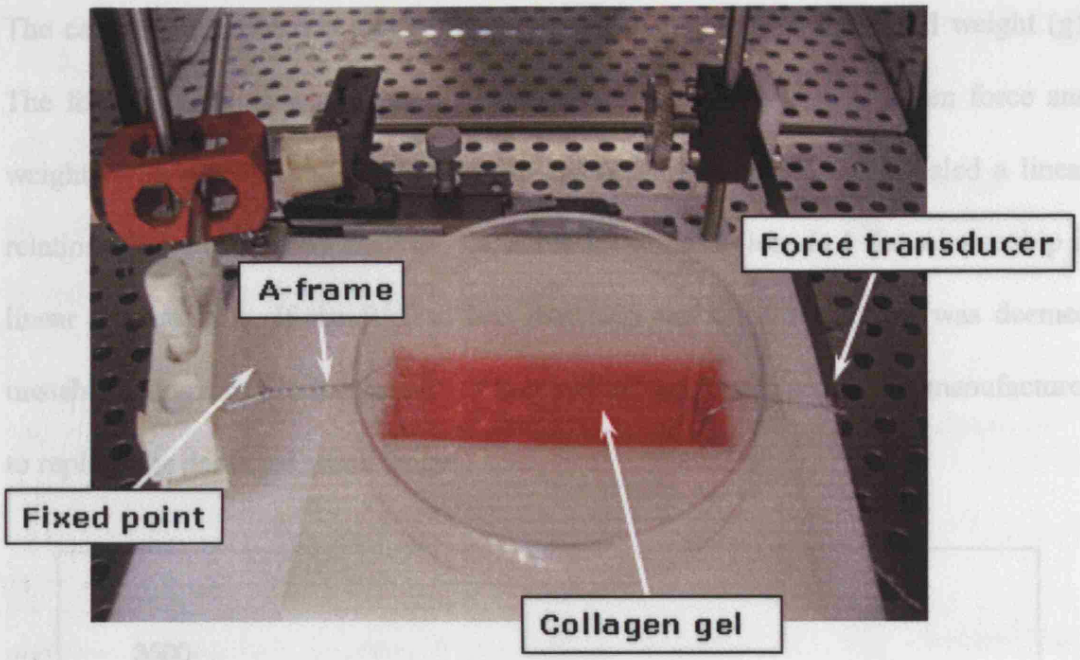

Figure 11: The CFM instrument set up is shown in this picture. The cell seeded collagen gel is tethered between a fixed point and a displacement force transducer. The CFM is used in a humidified, 37°C, CO_2 incubator. The cell-mediated collagen contraction is converted into a force reading via the force transducer and an external microprocessor.

Force Transducer Calibration

To ensure stable operation the CFM was calibrated regularly. The force transducer from each machine was positioned horizontally, and was calibrated against a set of known weights. First readings were taken when no weight was applied to the beam, i.e. zero force. Then five known weights (29.4, 49, 196, 294 and 490g) were placed consecutively on the hook at the tip of the transducer. The weights caused displacement of the Cu-Berrylium beam which was converted into a corresponding force, as described earlier. Force readings were recorded for 5 minutes for each mass.

The calibration curve was plotted as average force (μN) against applied weight (g). The force transducer was operating normally if the relationship between force and weight is linear. Adding a trend line to the acquired data points revealed a linear relationship by regression analysis, i.e. if the R^2 value is close to 1 the relationship is linear (Figure 12). If the R^2 was less that 0.95 the force transducer was deemed unstable and unsuitable for use. The Cu-beryllium beam was sent to the manufacturer to replace the damaged strain gauge.

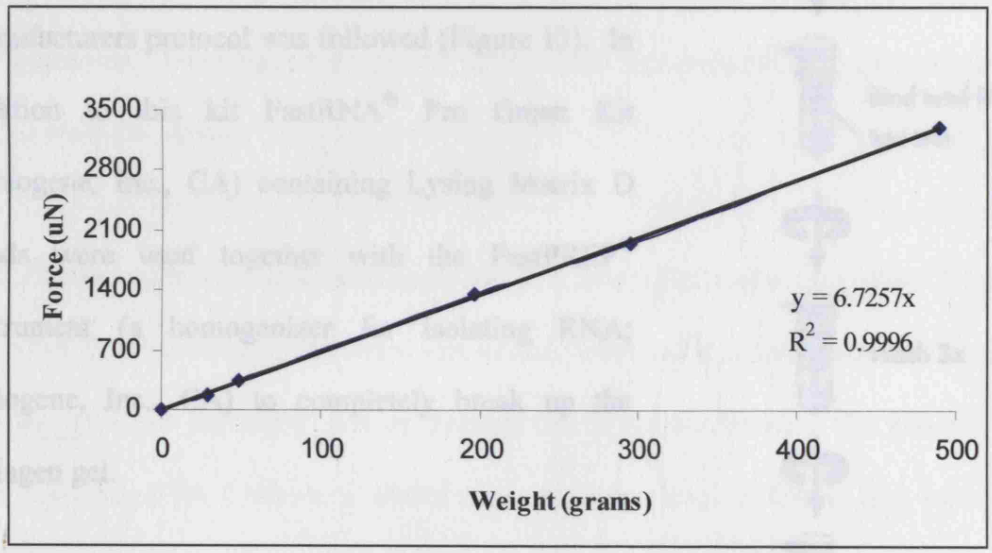

Figure 12: A typical force transducer calibration curve plotted as force (μN) against weight (grams). The displacement of the force transducer following the application of known weights enabled the calibration of the CFM.

3. RNA Extraction

All CFM experiments ran for 24 hours. The collagen gels were then flash frozen in liquid nitrogen and stored at -80°C to prevent RNA degradation.

The Qiagen RNeasy® (Qiagen, UK) procedure is an established technology for RNA purification and the manufacturers protocol was followed (Figure 13). In addition to this kit FastRNA® Pro Green Kit (Qbiogene, Inc., CA) containing Lysing Matrix D beads were used together with the FastPREP® instrument (a homogenizer for isolating RNA; Qbiogene, Inc., CA) to completely break up the collagen gel.

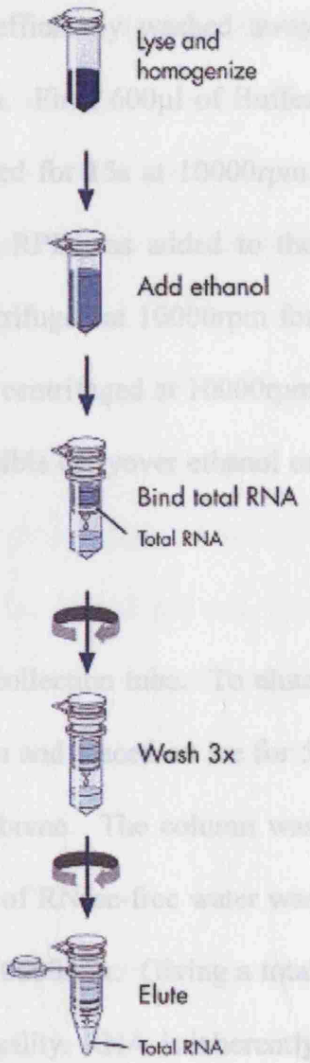

Figure 13: RNA extraction
(Source: RNeasy® Mini Handbook, Qiagen)

The cell-seeded collagen gels were then lysed in the presence of a highly denaturing guanidine-thiocyanate containing buffer (RLT). 1000µl of RLT buffer was added to the collagen gel in the Lysing Matrix D tube. The tube was processed in the FastPrep® instrument for 40 seconds at 6.0ms^{-1} then allowed to cool on ice for 5 minutes. The tube was returned to the instrument for a further 40 seconds at 6.0ms^{-1}, then removed and centrifuged at 13000rpm for 5 minutes. 1000µl of the homogenised supernatant was added to an equal volume of Ethanol and mixed well by pipetting. Ethanol was added to provide appropriate binding conditions. 600µl of the sample was transferred to an RNeasy

spin column and centrifuged at 10000rpm for 15s. The RNeasy Mini spin contains a silica based membrane which binds total RNA. The flow through was discarded and successive aliquots were centrifuged. Contaminants are efficiently washed away using a specialized high-salt buffer (RW1 and RPE) system. First, 600µl of Buffer RW1 was added to the RNeasy spin column and centrifuged for 15s at 10000rpm. Again the flow through was discarded. The next buffer, RPE was added to the RNeasy spin column in a new 2ml collection tube and centrifuged at 10000rpm for 15s. Followed by a second wash with RPE buffer this time centrifuged at 10000rpm for 2 minutes. The longer time period ensured that any possible carryover ethanol or buffer was eliminated.

The RNeasy spin column was then placed in a new 1.5ml collection tube. To elute the RNA 30µl of RNase-free water was added to the column and placed on ice for 5 minutes to allow the water to soak through the silica-membrane. The column was then centrifuged for 1 minute at 10000 rpm. A further 20µl of RNase-free water was added to the column and again centrifuged for 1 minute at 10000rpm. Giving a total of 50µl eluted, of which 3µl was used to measure RNA quality. RNA is inherently fragile and therefore subject to degradation process during preparation and handling, therefore the integrity of extracted RNA was monitored using the Agilent 2100 Bioanalyser (analyses quality and quantity of RNA; Agilent, US) together with the RNA 6000 Nano LabChip® Kit (Agilent, US). The manufacturers' protocol were carefully followed, including decontaminating the electrodes, preparing the gel dye mix, loading the gel dye mix and loading the RNA 6000 Nano LabChip® onto the Biolanalyser.

1. *Decontaminating the Electrodes*

Before each use the electrodes of the bioanalyser were decontaminated. The electrode cleaner provided was filled with 350µl of RNaseZAP® and placed in the bioanalyser, the lid was closed and left for 1 minute. The electrode cleaner was removed and a second electrode cleaner filled with 350µl of RNase-free water was placed in the machine the lid was closed and left for 10 seconds. The second cleaner was removed and the electrodes were now decontaminated and ready for use.

2. Preparing the Gel-Dye Mix

550µl of RNA 6000 Nano gel matrix was placed into the top receptacle of the spin filter and centrifuged at 4000rpm for 10 minutes. 65µl of the filtered gel was then added to 1µl of RNA 6000 Nano dye concentrate in a 0.5ml RNase-free microfuge tube and vortexed thoroughly.

3. Loading the Gel-Dye Mix

A new RNA Nano chip was taken out of it's sealed bag and placed in the Chip Priming Station (need to go to website for picture). The Chip Priming Station is used to dispense the gel-dye mix by pressurization. 9µl of the gel-dye mix was pipetted into the well marked 'g' and the priming station is closed. The plunger was then pressed until it was held in position by the syringe clip for exactly 30 seconds. The plunger was then released with the clip release mechanism. After 5 seconds the plunger is slowly pulled back into the starting position. 9µl of the gel-dye mix were also pipetted into the other wells marked 'g'. The remaining gel-dye mix was discarded.

4. Loading the RNA 6000 Nano Marker, Ladder and Samples

5µl of the RNA 6000 Nano Marker was then pipetted into each of the 12 sample wells as well as the well marked with the ladder symbol. The ladder was heat denatured at 70°C for 2 minutes, then 1µl of the ladder was pipetted into the well marked with the ladder. Similarly, 1µl of each RNA sample was pipetted into each of the 12 sample wells. The chip was then placed in the adapter of the vortex mixer and mixed for 1 minute at 2400rpm. The chip was then placed into the bioanalyser where the electrodes in the cartridge fit into the wells of the chip. The 2100 expert software showed that the chip was inserted correctly and proceeded to run the analysis, the output was an electropherogram summary (Figure 14 (a)). Only high quality RNA (ratio in the range 1.6-2.0) was used for subsequent cDNA synthesis.

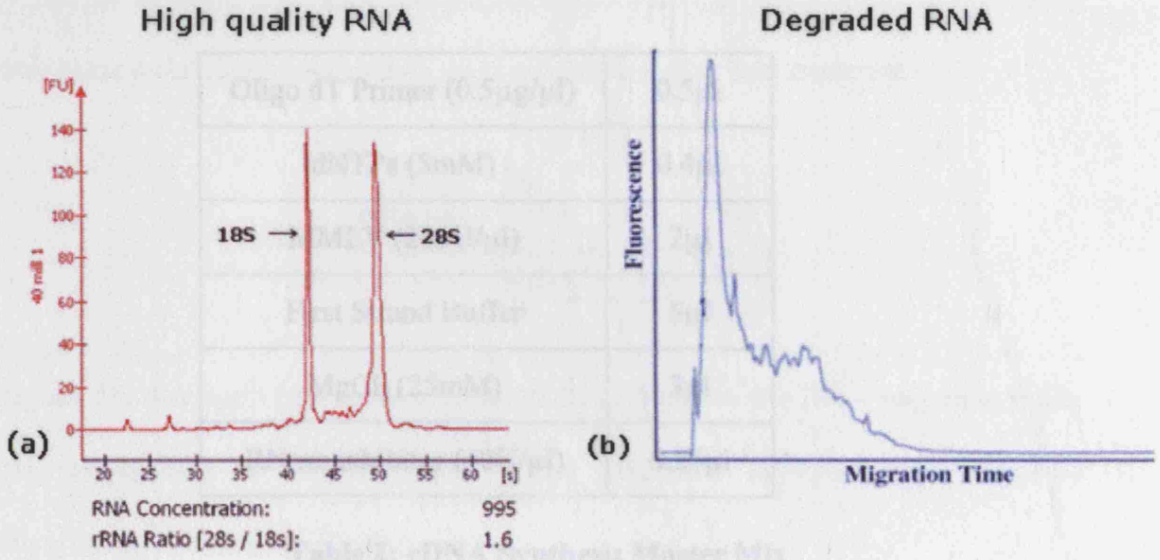

Figure 14: Example Electropherogram Summary. (a) High quality RNA demonstrated 2 intact RNA species, the 18S and 28S ribosomal RNAs. RNA concentration and quality were determined by the ratio of 18S and 28S. (b) An extreme example of degraded RNA (due to contamination or handling error) is

also shown (Source: http://www.primerdesign.co.uk/RNA_quality_control-_technical_bulletin.asp)

4. cDNA Synthesis

RNA was reverse transcribed into cDNA using Expand Reverse Transcriptase (Roche Diagnostics, East Sussex, UK). This step synthesised a complementary strand of DNA to the RNA in the sample. For cDNA synthesis a master mix was prepared containing; oligodeoxythymidylic primer (Oligo dT Primer), deoxynucleoside triphosphates (dNTPs), Moloney Murine Leukemia Virus (MMLV reverse transcriptase), Magnesium Chroride ($MgCl_2$), first strand buffer and RNase inhibitor, according to volumes shown in Table 1.

Oligo dT Primer (0.5μg/μl)	0.5μl
dNTPs (5mM)	0.4μl
MMLV (200U/μl)	2μl
First Strand Buffer	5μl
$MgCl_2$ (25mM)	3μl
RNase inhibitor (40U/μl)	0.25μl

Table 2: cDNA Synthesis Master Mix

The primers bind to target sites and dNTP's provide the additional bases required to produce the complementary strand. The MMLV reverse transcriptase is the enzyme that synthesises first strand cDNA. 11.15μl of the master mix was added to a 0.5ml eppendorf tube and 2μg of RNA was added to the appropriate amount of RNase-free

water to give a total reaction mix of 50µl. A drop of molecular grade mineral oil (Sigma, Dorset, UK) was added on top of the mix and the lid gently closed. The thermo-cycler (Hybaid OMN-E) was programmed according to the sequence: 25°C for 10 minutes, 42°C for 60 minutes and 94°C for 5 minutes (to denature the RT enzyme).

5. Polymer Chain Reaction (PCR)

PCR amplifies specific DNA sequences. This technology is based on the use of DNA polymerase to copy a DNA sequence in repeated cycles of replication. Each cycle doubles the amount of DNA synthesised in the previous cycle.

Taq polymerase is a thermostable DNA polymerase isolated from the thermophilic bacterium Thermus aquaticus; this polymerase is stable at much higher temperatures than eukaryotic DNA polymerases, so is not denatured by heat treatment.

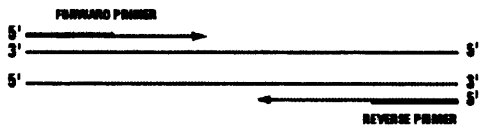

Figure 15: Forward and reverse primers hybridized to the DNA sequence guide the polymerase to the sequence to be amplified (adapted from Applied Biosystems® Chemistry Guide).

The polymerase is guided to the sequence to be copied by short primer oligonucleotides (relatively short single-stranded nucleic-acid chains usually consisting of up to approximately 20 nucleotides) that are hybridized to the DNA template at the beginning (forward primer) and end (reverse primer) of the desired

DNA sequence (Figure 15). These primers are designed so that they provide a primer for DNA replication on each strand of the original double stranded DNA to be amplified (Alberts *et al*, 1998).

PCR is extremely sensitive it can detect a single copy of a DNA sequence in a sample by amplifying it so much that it becomes detectable. Using this technique a given nucleotide sequence can be selectively and rapidly replicated in large amounts from any DNA that contains it.

Real-time PCR is the ability to monitor the progress of the PCR as it occurs rather than at the end of the reaction. The development of fluorogenic labeled probes enabled the detection of only specific PCR product as they accumulated during the reaction.

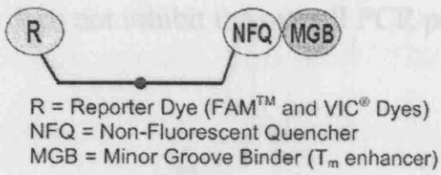

Figure 16: TaqMan fluorogenic labeled probe. An oligonucleotide probe is constructed containing a reporter dye at the 5' end, a minor-groove-binder moiety and a non-fluorescent quencher dye on the 3´ end (adapted from Applied Biosystems® Chemistry Guide).

The TaqMan (Applied Biosystems®) probes used in this study are fluorogenic-labelled probes (Figure 16). While the probe is intact, the proximity of the quencher dye greatly reduces the fluorescence emitted by the reporter dye by fluorescence resonance energy transfer (FRET) through space.

If the target sequence is present, the probe anneals downstream from one of the primer sites and is cleaved by the 5′ nuclease activity of Taq DNA polymerase as this primer is extended.

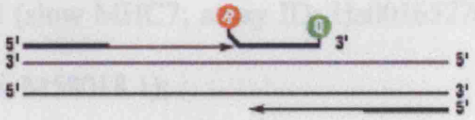

Figure 17: A fluorescent reporter (R) dye and a quencher (Q) are attached to the 5' and 3' ends of the TaqMan® probe. When the probe is intact, the reporter dye emission is quenched (adapted from Applied Biosystems® Chemistry Guide).

This cleavage of the probe separates the reporter dye from the quencher dye, increasing the reporter dye signal (Figure 18). Further, it removes the probe from the target strand, allowing primer extension to continue to the end of the template strand, i.e. inclusion of the probe does not inhibit the overall PCR process.

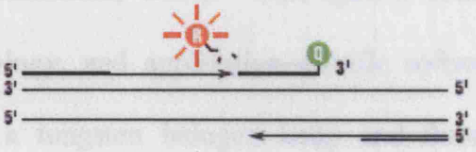

Figure 18: During each extension cycle the DNA polymerase cleaves the reporter dye from the probe. Once separated from the quencher the reporter dye emits its characteristic fluorescence (adapted from Applied Biosystems® Chemistry Guide).

Additional reporter dye molecules are cleaved from their respective probes with each cycle, resulting in an increase in fluorescence intensity proportional to the amount of amplicon (synthesised segments of DNA) produced.

The specific probes selected for this study were:

1. Myogenin (assay ID: Hs01072232_m1; GenBank mRNA sequence: X17651.1);
2. Myosin Heavy Chain (MyHC) two isoforms:
    a. MyHC-β (slow MHC7; assay ID: Hs00165276_m1; GenBank mRNA sequence: M58018.1);
    b. MyHC-IIX/C (fast MHC1; assay ID: Hs00428600_m1; GenBank mRNA sequence: AF111785.1);
3. MMP-2 (gelatinase A,72kDa type IV collagenase; assay ID: Hs00234422_m1; GenBank mRNA sequence: J03210.1); and
4. GAPDH (glyceraldehyde-3-phosphate dehydrogenase; assay ID: Hs99999905_m1; GenBank mRNA sequence: M33197.1).

These probes were optimised to universal cycling conditions by Applied Biosystems®.

RT-PCR was performed using Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, California, USA). This system combines thermal cycling, fluorescence-dye technology, and application-specific software. All sample wells were illuminated with a tungsten halogen lamp and fluorescence emission was detected through four filters to a camera.

The universal thermal cycling parameters presented in Table 3 were used for the reaction. This protocol was designed by Applied Biosystems® for use with all assays designed according to Applied Biosystems assay design guidelines i.e. no optimization of the thermal cycling parameters was necessary for probes used.

Times and Temperatures			
Initial Steps		PCR (40 Cycles)	
AmpErase UNG Activation	AmpliTaq Gold DNA Polymerase Activation	Melt	Anneal/Extend
HOLD	HOLD	CYCLE	
2 min @ 50 °C	10 min @ 95 °C	15 sec @ 95 °C	1 min @ 60 °C

Table 3: Thermal cycling parameters for quantification assays when using TaqMan Universal PCR Master Mix

PCR products are detected cycle-by-cycle in a single reaction tube. The components of this reaction are listed in Table 4 below. The TaqMan® Universal PCR Master Mix (Applied Biosystems, California, USA) contained AmpliTaq Gold® DNA polymerase, AmpErase® UNG, dNTPs with dUTP.

Reaction Component	Volume/Well (25µl Reaction)
TaqMan® Universal PCR Master Mix	12.5
20× Assays-on-Demand™ Gene Expression Assay Mix	1.25
cDNA diluted in RNase-free water	11.25
Total	25

Table 4: Components of the PCR reaction

Data is collected throughout the PCR process, rather than at the end. Compared with manual PCR quantitation techniques, RT-PCR has greater sensitivity and precision. RT-PCR is characterised by the point in time during cycling when amplification of a target is first detected rather than the amount of target accumulated after a fixed number of cycles. An amplification plot graphically displays the fluorescence detected over the number of cycles that were performed (Figure 19).

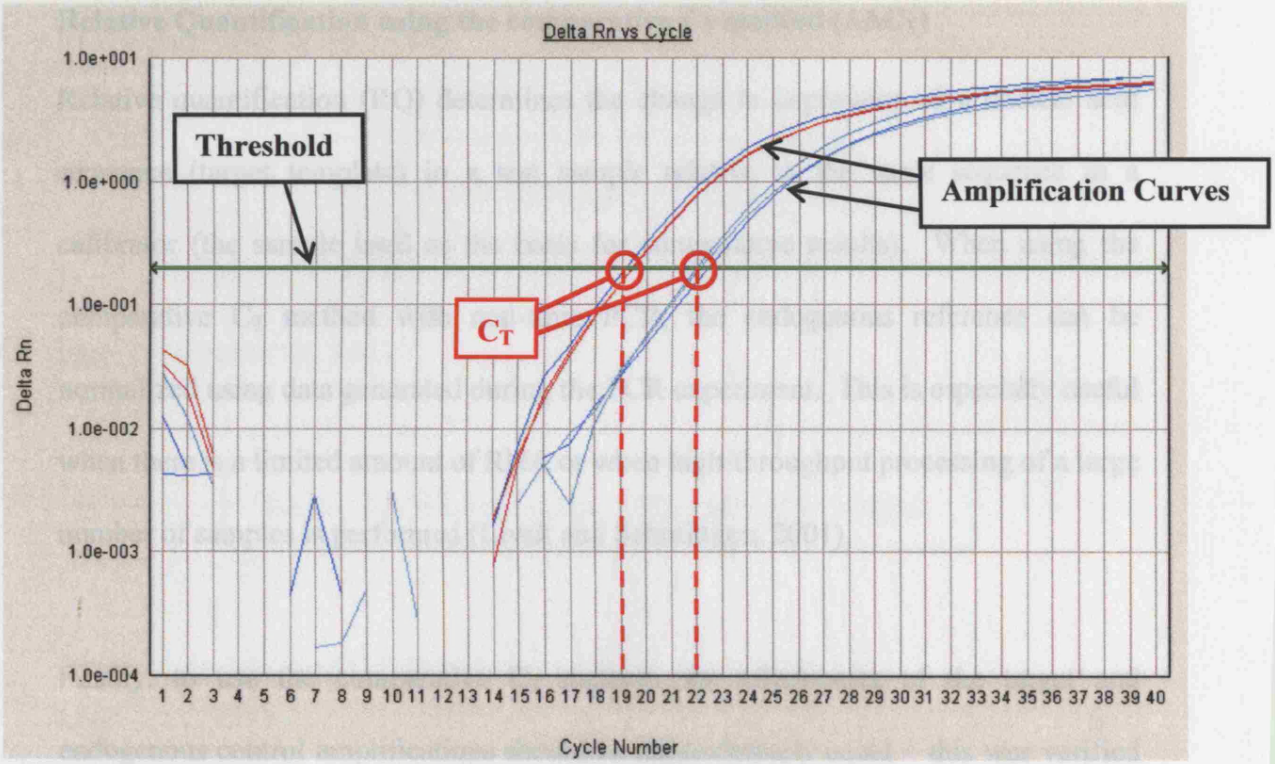

Figure 19: Data is collected throughout the Real Time PCR process and the fluorescence detected is graphically displayed over the number of cycles that were performed (amplification curves). The threshold at which a sufficient amount of target is amplified and the corresponding cycle numbers (C_T) are also highlighted.

In the initial cycles of PCR, there is no significant change in fluorescence signal. This predefined range of PCR cycles is called the "baseline". The Sequence Detection

Software (SDS®) generates a baseline subtracted amplification plot by calculating a mathematical trend using R_n (the emission intensity of the reporter dye) values corresponding to the baseline cycles. Then an algorithm searches for the point on the amplification plot at which the delta ΔR_n value crosses the threshold. The threshold cycle or C_T value is the cycle at which a statistically significant increase in ΔR_n is first detected.

Relative Quantification using the comparative C_T method ($\Delta\Delta C_T$)

Relative quantification (RQ) determines the change in expression of a nucleic acid sequence (target template) in a test sample relative to the same sequence in a calibrator (the sample used as the basis for comparative results). When using the comparative C_T method with real-time PCR, the endogenous reference can be normalized using data generated during the PCR experiment. This is especially useful when there is a limited amount of RNA or when high-throughput processing of a large number of samples is performed (Livak and Schmittgen, 2001).

Finally, to use the comparative C_T method, the efficiencies of the target and endogenous control amplifications should be approximately equal – this was verified by Applied Biosystems®. The advantage of using the comparative CT method is that the need for a standard curve is eliminated. This increases throughput and also eliminates the adverse effect of any dilution errors made in creating the standard curve samples.

Therefore, the comparative C_T method was used. The amount of target (eg. MMP2) normalised to an endogenous reference (GAPdH) and relative to a calibrator is given by the equation:

$$2^{-\Delta\Delta C_T}$$

The $\Delta\Delta C_T$ method was used to calculate the fold differences in the target genes between samples. Four steps were used to perform this analysis.

Step 1: Calculate the average C_T

Step 2: Calculate the ΔC_T value

$$\Delta C_T = C_{T\ target} - C_{T\ reference}$$

Step 3: Calculate the $\Delta\Delta C_T$

$$\Delta\Delta C_T = \Delta C_T \text{ test sample} - \Delta C_T \text{ calibrator sample}$$

Step 4: Calculate the RQ fold increase

$$2^{-\Delta\Delta C_T}$$

For example,

Experiment	Average C_t		ΔC_t	$-\Delta\Delta C_t$	RQ
	Target Gene (MMP2)	Endogenous Control (GAPDH)	C_t (MMP2) - C_t (GAPDH)	$-(\Delta C_{t,x} - \Delta C_{t,\text{ calibrator}})$	$2^{-\Delta\Delta Ct}$
Untreated sample	24.310	19.304	5.006	2.856	7
Treated sample	21.692	20.068	1.624	6.238	75
Calibrator	27.068	19.206	7.862	0	1

Table 5: Data analysis using C_t values obtained from PCR. The comparative method was used to calculate the relative gene expression.

The average C_T was obtained from the PCR run. The ΔC_T value was determined by subtracting the average GAPDH C_T from the average MMP2 C_T value. From Table 5,

ΔC_T treated = 21.692 – 20.068 = 1.624

$-\Delta\Delta C_T$ was calculated by subtracting the ΔC_T calibrator value from the ΔC_T target value. From Table 5,

$$-\Delta\Delta C_T = -(\Delta C_T \text{ treated} - \Delta C_T \text{ calibrator})$$

$$= -(1.624 - 7.862) = 6.238$$

The RQ for the 'treated' relative to calibrator was calculated using the equation: $2^{-\Delta\Delta Ct}$. Following this example through: $2^{6.238} = 75$. Indicative of a relative up-regulation of the gene in the treated sample compared to the gene expression in the untreated sample.

6. Statistical Analysis

Statistical analysis of data (minimum of n=3 for all the experiments) were performed using the non-parametric, ANOVA (analysis of variance) testing (Kruskal-Wallis), using GraphPad Prism (Graphpad Software, San Diego, USA). Briefly, the purpose of ANOVA was to test for significant differences between means. If only two means are being compared, ANOVA will give the same results as the t-test.

Chapter 1: Cell separation and markers of myogenic differentiation in 2D

Introduction

To date, the *in vitro* 2D culture and differentiation of skeletal muscle myoblasts has been an invaluable technique to explore many facets of skeletal muscle physiology and pathology, providing an isolated myogenic system, free from the complexity of whole animal studies (Cooper *et al.*, 2004). Upon enzymatic dissociation single mononuclear cells are able to migrate out from scissor-minced biopsies of human craniofacial masseter muscle (Mouly *et al.*, 1993; Sinanan *et al.*, 2004). These mononuclear cells are a heterogenous mixture of myogenic (giving rise to or forming muscle) and non-myogenic cells (Yaffe and Saxel, 1977; Blau and Webster, 1981; Stewart et al., 2003; Cooper et al., 2004; Sinanan et al., 2004). This heterogenous mixture of cells is due to the amount and distribution of connective tissue which results in the inevitable combination of primary myoblast cultures with other cells (i.e. fibroblasts) (Yaffe and Saxel, 1977).

The ratio of myogenic to non-myogenic cells in primary cell cultures has been demonstrated to influence the muscle cells present, making it difficult to distinguish between the properties intrinsic to the myogenic cells and the effects exerted by other cell types (Yaffe and Saxel, 1977; Blau and Webster, 1981). Webster et al. (1988) reported that the presence of fibroblasts (non-myogenic cells) inhibited the biochemical differentiation of satellite cells. On the other hand, Cooper et al. (2004) recently reported that primary myoblasts cultures that are contaminated by fibroblasts

fared better in culture than do C2C12 cells, suggesting that fibroblasts secrete extracellular matrix components and growth factors that may contribute to the survival and differentiation of myoblasts. The purity of the cell population is important (Bonavaud et al., 2002; Sinanan et al., 2004) and little attention has been paid to the interactions between muscle fibres, myogenic cells and other (non-myogenic) cells such as fibroblasts (Singh et al., 2000).

The ability to obtain large homogenous cell populations which undergo differentiation in controlled culture conditions would enable comparative studies at the cellular and biochemical levels of the intrinsic properties of myogenic cells obtained from muscle biopsies. To this end a number of researchers have reported several myoblast purification procedures including density centrifugation, selective adhesion, fluorescence activated cell sorting (FACS), size separation based flow cytometry and chemical use of fibroblast growth inhibitors (Kao and Prockop, 1977; Yablonka-Reuveni *et al.*, 1987; Webster et al., 1988; Baroffio *et al.*, 1993; Blanton *et al.*, 1999 Immuno-magnetic techniques have recently been reported (Lequerica *et al.*, 1999; Sinanan *et al.*, 2004; Park *et al.*, 2006) to purify myoblasts from primary cultures by magnetic activated cell sorting (MACS) with good yields. Sinanan *et al* (2004) reported a purified myoblast subpopulation which expressed desmin in at least 95% of the cells. Desmin is a cytoplasmic intermediate filament protein that is expressed by myogenic myoblasts and myotubes (Kaufman and Foster, 1988; Lewis *et al.*, 2000; Bonavaud et al., 2002).

Kaufman and Foster (1988) first noted that fibroblasts do not express desmin and this can be utilised as a way of distinguishing them from myoblasts *in vitro*. Desmin

labelling has been particularly instructive in identifying myoblasts because it is one of the first muscle-specific proteins to be produced in myoblasts (Lawson-Smith and McGeachie, 1998). Single desmin negative (non-myogenic) cells in muscle derived cultures are either (i) quiescent myoblasts that have not entered myogenic differentiation or (ii) infiltrating intramuscular fibroblasts (van der Ven *et al.*, 1992; Lewis *et al.*, 2001). Desmin was used in this study to determine the percentage of myogenic cells within the muscle derived cell cultures used for further experimentation.

The first aim of this study was to separate the heterogenous mixture of mononuclear cells derived from craniofacial muscle into purified subpopulations of myogenic and non-myogenic cells using immuno-magnetic techniques (MACS®). CD56 was chosen for positive sorting as it recognizes a cell-surface epitope that is highly expressed on the target cells (and not expressed at all on the non-myogenic cells) and is non-labile under processing conditions (Sinanan *et al.*, 2004). CD56 is a cell adhesion molecule (CAM), also known as antigen Leu-19 (leucocyte differentiation antigen 19) that is expressed constitutively in proliferating myoblasts (Illa *et al.*, 1992; Belles-Isles *et al.*, 1993). Other cells also express CD56, including certain lymphocytes and neurons, but not fibroblasts (Stewart *et al.*, 2003).

The central event in skeletal muscle development is the fusion (differentiation) of myoblasts to form multinucleated myotubes (Lewis *et al.*, 2000). This phenomenon can be exploited *in vitro*. Myogenic MDCs isolated from the human masseter muscle, grown under permissive culture conditions can fuse and form myotubes (Sinanan *et al.*, 2004). The onset and progression of fusion of myoblasts *in vitro* is controlled by

a set of complex interactions between myoblasts and their environment (Melo *et al.*, 1996). The mononucleated cells undergo a series of events including cell fusion and the synthesis of a new set of proteins, such as α-sarcomeric actin an established marker of terminal differentiation (Sinanan *et al.*, 2004). *The second aim of this study was to test that myogenic cells fuse to form multinucleated myotubes in 2D and express α-sarcomeric actin.*

The terminal differentiation of myogenic cells begins with their withdrawal from the cell cycle. It remains unclear what the stimulus for fusion is and how these events are regulated (Schultz and McCormick, 1994). Of particular interest however, is the expression of myogenin mRNA observed when myoblasts were induced to differentiate (Melo *et al.*, 1996). Myogenin is a DNA binding transcriptional regulatory protein. Work both *in vitro* and *in vivo* has found that myogenin is up-regulated during the differentiation of skeletal myoblasts (Sassoon *et al.* 1989, Wright *et al.* 1989). Nabeshima *et al.* (1993) investigated the role of myogenin in mice by disrupting the myogenin gene, their results showed normal numbers of proliferating myoblasts, however they failed to differentiate, concluding that myogenin may have a role in the transition from myoblasts to differentiated myotubes. Myogenin is well established as a secondary myogenic regulatory protein expressed prior to fusion of myoblasts to form myotubes (Rawls *et al.* 1995). *The final aim of this part of the study was to test whether multinucleated 2D cell cultures express myogenin. The objective was to quantitate myogenin mRNA expression in cultures containing single cells and multinucleated myotubes using quantitative RT-PCR.*

Materials and Methods

Cell Separation

The primary human craniofacial muscle derived cells (MDC) that migrate off the biopsy are a mixture of myogenic and non-myogenic cells. In order to obtain a purified myogenic cell population from the mixed population of primary MDCs, a magnetic sorting system was selected. MACS® is a magnetic separation technology designed to magnetically separate cells in suspension according to cell surface antigens. It has been shown that the super-paramagnetic MACS® MicroBeads at about 50nm diameter do not significantly change the scatter properties of the cell or their light microscopic appearance. Further, they are biodegradable due to their small size and biochemical composition (iron oxide and polysaccharide), degrading during subsequent culture such that cell function and viability were preserved.

Principals of MACS® separation

First the target cells are magnetically labeled and then the cell suspension is loaded into a MACS® Column which is placed in the magnetic field of the MACS® separator (Figure 20).

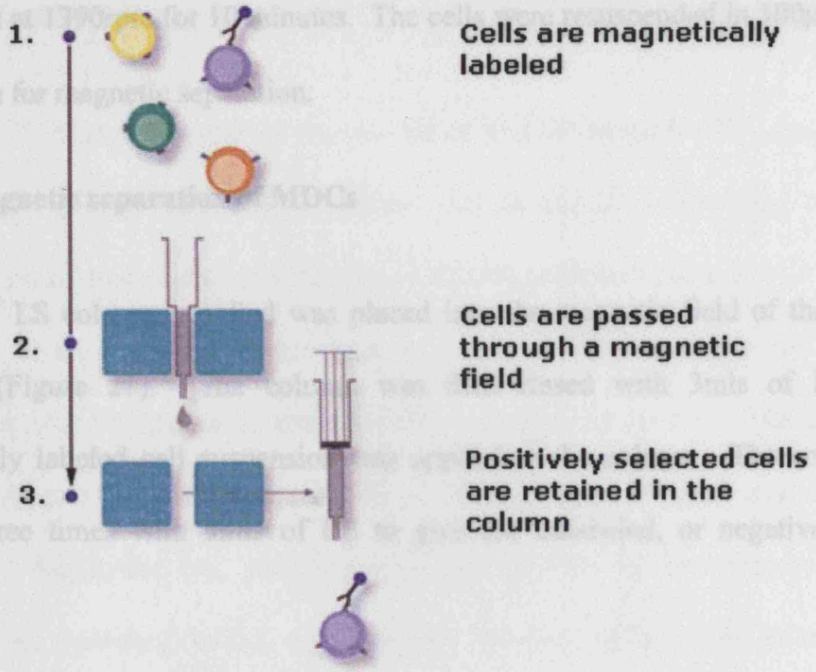

Figure 20: Schematic showing the principles of MACS® separation. First the cells are magnetically labeled with the antigen specific MicroBead (CD56+) and then the cells are passed through a magnetic field. The positively (magnetically labeled) selected cells are retained in the column isolated from the original mixture of cells.

1. **Magnetic labeling of myogenic cells (CD56+)**

MDCs were magnetically labelled according to the manufacturers protocol (Miltenyi Biotech, USA). Phosphate buffered saline pH 7.2, was supplemented with 0.5% bovine serum albumin (Sigma, Poole, UK) and 2mM EDTA. This specially prepared phosphate buffer (PB) was degassed using a vacuum pump. The mixture of myogenic and non-myogenic cells was suspended in 80µl of PB and 20µl of anti-human CD56+ MicroBeads per 10^7 total cells, mixed well and allowed to incubate for 15 minutes at 4–8°C. The cells were then washed in 1-2mls of PB per 10^7 total cells and

centrifuged at 1390rpm for 10 minutes. The cells were resuspended in 500µl of PB in preparation for magnetic separation.

2. Magnetic separation of MDCs

A MACS® LS column supplied was placed into the magnetic field of the MACS® separator (Figure 21). The column was then rinsed with 3mls of PB. The magnetically labeled cell suspension was applied to the column. The column was washed three times with 3mls of PB to give the unlabeled, or negative (CD56-) fraction.

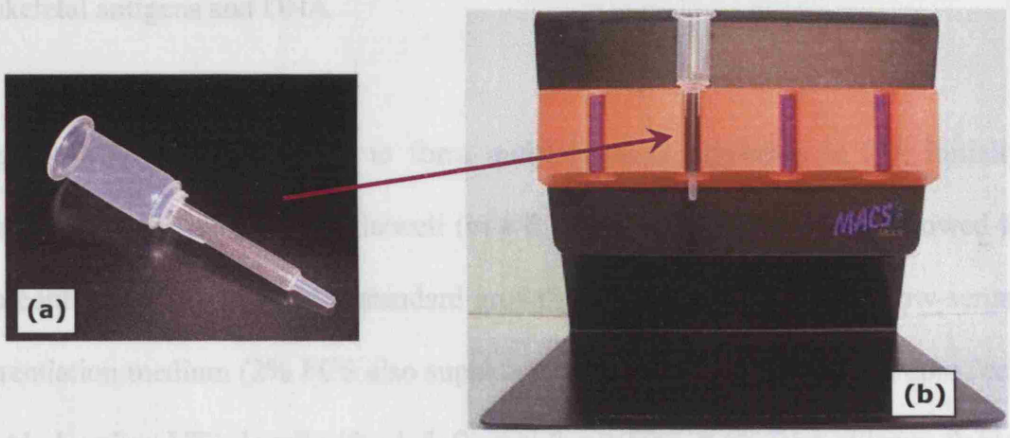

Figure 21: Picture showing the magnetic separation apparatus. The (a) MACS® column was placed into the magnetic field of the (b) MACS® Separator.

The column was then removed from the magnetic field and the positive fraction (magnetically labeled cells; CD56+) was flushed out of the column by flowing 5mls of PB through the column and firmly applying the plunger supplied with the column. Both the labeled (myogenic) and unlabeled (non-myogenic) fractions were used for subsequent culture and studies.

Immunostaining

The purity (% myogenic cells) of the co-culture and the magnetically separated cells was evaluated using immunohistochemistry and fluorescent microscopy techniques. The single cells were plated at a density of 25,000 cells/well (in a 6 well plate) and fixed after 24hours. Cells were fixed with 2% paraformaldehyde for 5-10 min followed by post-fixation with ice-cold 20% methanol for 10 min. The aim of cell fixation is to preserve cell structure and to present target antigens available to the antibodies. Aldehydes (eg. paraformaldehyde) fix cells by cross-linking proteins. Alcohols (eg. methanol) effect rapid fixation by dehydration, lipid extraction and protein precipitation. Alcohols are the preferred fixative in the combined analysis of cytoskeletal antigens and DNA.

Myogenic MDCs were induced to form multinucleated myotubes in 2D; initially seeded at a density of 25,000 cells/well (in a 6 well plate), the cells were allowed to reach confluence at which point standard growth medium was changed to low-serum differentiation medium (2% FCS also supplemented with IGF-1 (10ng/ml; PeproTech EC Ltd., London, UK), Insulin (5ng/ml; Sigma, Poole,UK), 0.5% BSA (Sigma, Poole, UK) and DMEM) to stimulate incorporation of myoblasts into multinuclear myotubes (Sinanan *et al.*, 2004), which were identified by optical microscopy. Myotubes were similarly fixed with 2% paraformaldehyde for 5-10 min followed by post-fixation with ice-cold methanol for 10 min.

The single cells and myotubes were then detergent permeabilized with 0.25% Triton X-100 (Sigma, Poole, UK) in antibody diluting solution (ADS; phosphate buffered saline, 10% inactivated FCS (@60°C for 30 minutes), 0.0625% NaN_3, 0.1M lysine

(Sinanan *et al.*, 2004)) for 10-15 minutes. Detergents can dissolve the cell membranes by forming detergent-lipid and detergent-lipid-protein mixed micelles. The single cells were incubated overnight (room temperature) with the skeletal muscle specific primary antibody: mouse monoclonal anti-human desmin (Clone: D33, Isotype: IgG, Dako, Glostrup, Denmark) which was diluted 1:2000 in ADS. Myotubes were further incubated overnight with anti-α-sarcomeric actin (Clone: 5C5, Isotype: IgM Sigma-Aldrich, Poole, UK) which was diluted 1:12000 in ADS.

After overnight incubation, single cells and myotubes were washed in PBS and incubated with the secondary antibodies. Anti-mouse IgG class specific FITC was diluted 1:200 in ADS and anti-mouse IgM specific Cy3 diluted 1:1000 in ADS for 30 minutes at room temperature, enabling visualisation of desmin and α-sarcomeric actin respectively. Nuclei were identified using the fluorescent DNA binding probe DAPI (4',6-diamidino-2-phenylindole; 1.0 ng/ml; Sigma–Aldrich, Poole, UK) which was diluted 1:10,000 in ADS. The coverslips were washed in PBS and were mounted with the glycerol-based anti-fade media, Citifluor^{TM} (Citifluor Ltd., London, U.K.) and sealed with clear nail varnish.

The samples were visualised using an inverted Leica DMIRB microscope; cell-associated fluorescence was visualised with epifluorescence using the following filters: FITC (narrow band excitation at 485nm and emission at 520nm), Cy3 (excitation at 550nm and emission at 570 nm) and DAPI (UV excitation at 372nm and emission at 456nm). The cells were imaged with a ×63-oil immersion lens and captured using Leica FW4000 image-processing software (Shah *et al.*, 2005).

The proportion of myogenic cells in the primary heterogenous mixture of MDCs and the separated myogenic and non-myogenic subcultures, were determined for at least ten fields (n=10+) for each sample (n=3), which were viewed in a systematic manner. The total number of stained nuclei (cells) was counted in each field (Shah *et al.*, 2005). Within the same field of view, cells which expressed desmin were also counted. The proportion of myogenic cells was calculated as the number of cells expressing desmin over the total number of cells in the field of view according to the equation:

$$Proportion\ (\%)\ myogenic\ cells = \frac{no.\ cells\ expressing\ desmin}{total\ no.\ of\ stained\ nuclei}$$

This was averaged for the number of fields evaluated.

Results

The primary human craniofacial MDC cultures used in this study contained a mixture of myogenic and non-myogenic cells. These cells are indistinguishable in size and shape under phase contrast light microscopy (Figure 22A). Myogenic cells were distinguished from non-myogenic cells *in vitro* by their ability to express desmin (Figure 22B). Previous researchers have established the proportion of myogenic cells to non-myogenic cells in cultures derived from human masseter muscle biopsies, from more than 15 different donors, to range from 5 to 45% (Lewis et al., 2000; Singh et al., 2000; Sinanan et al., 2004). The proportion of myogenic cells within the heterogenous culture used in this study was 19 ± 4.9 % (n=10+) falling within the range of previously published results.

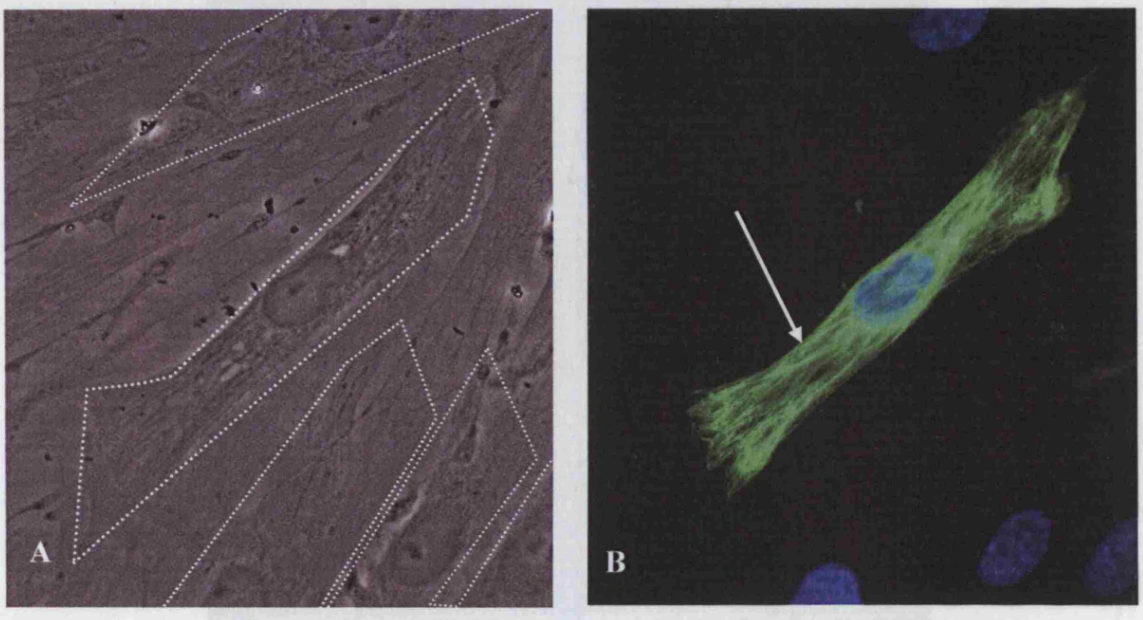

Figure 22: Primary human craniofacial MDCs are a heterogenous mixture of mononuclear cells that are indistinguishable from one another in size and shape under phase contrast (outlined with white dotted lines) (A) light microscopy (63×). Myogenic cells (B) are immunoreactive for desmin (green; indicated by

arrow). In contrast, non-myogenic cells are negative for desmin and are denoted by DAPI nuclear labelling (blue).

Skeletal muscle cells differentiate upon confluency, however fusion can be induced before myotubes become visible, by changing the high-nutrition culture medium to low-nutrition medium (ven der Van *et al.*, 1992; Mouly *et al.*, 1993; Sinanan *et al.*, 2004; Riboldi *et al.*, 2005). When the cells in this study were 70-80% confluent and the culture conditions were changed from standard growth medium to differentiation medium, some of the myogenic cells within the heterogenous MDC culture fused to form multinucleated myotubes.

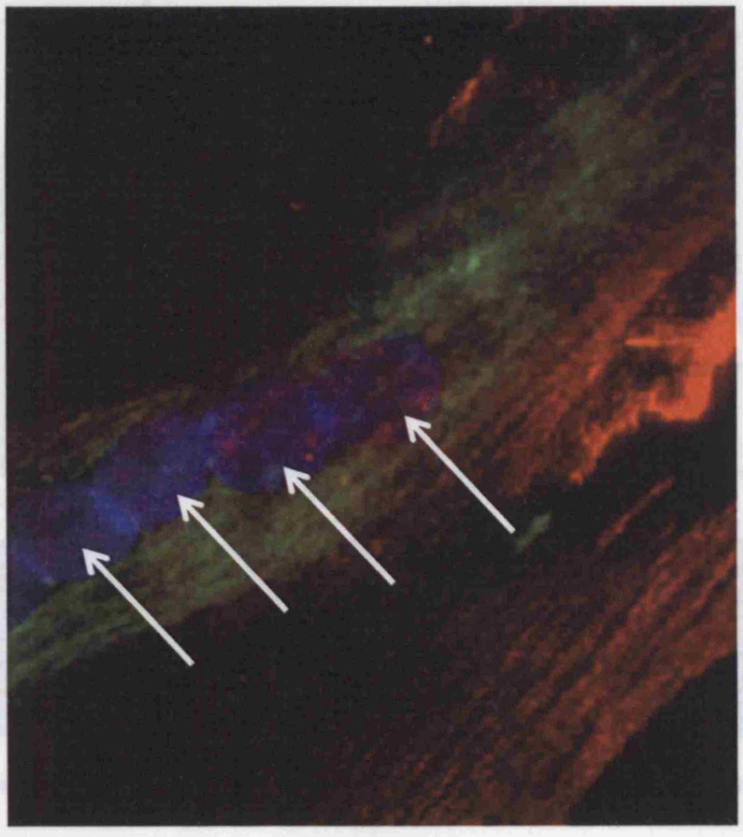

Figure 23: Myogenic primary human MDCs fuse to form multinucleated myotubes. This immuno-fluourescent micrograph depicts a multinucleated (blue; indicated by arrows) myotube that is immunoreactive for the cytoskeletal

protein desmin (green) and α-sarcomeric actin (red); α-sarcomeric actin is a marker of terminal differentiation in striated muscle (magnification ×63).

Fusion was defined as the formation of myotubes containing 3 or more nuclei. Myotubes were easily identified at the light microscope level both by the configuration of nuclei and their cellular components. These cultures immunostained positive for the cytoplasmic protein α-sarcomeric actin (Figure 23), an established marker of terminal differentiation in striated muscle (Sinanan *et al.*, 2004).

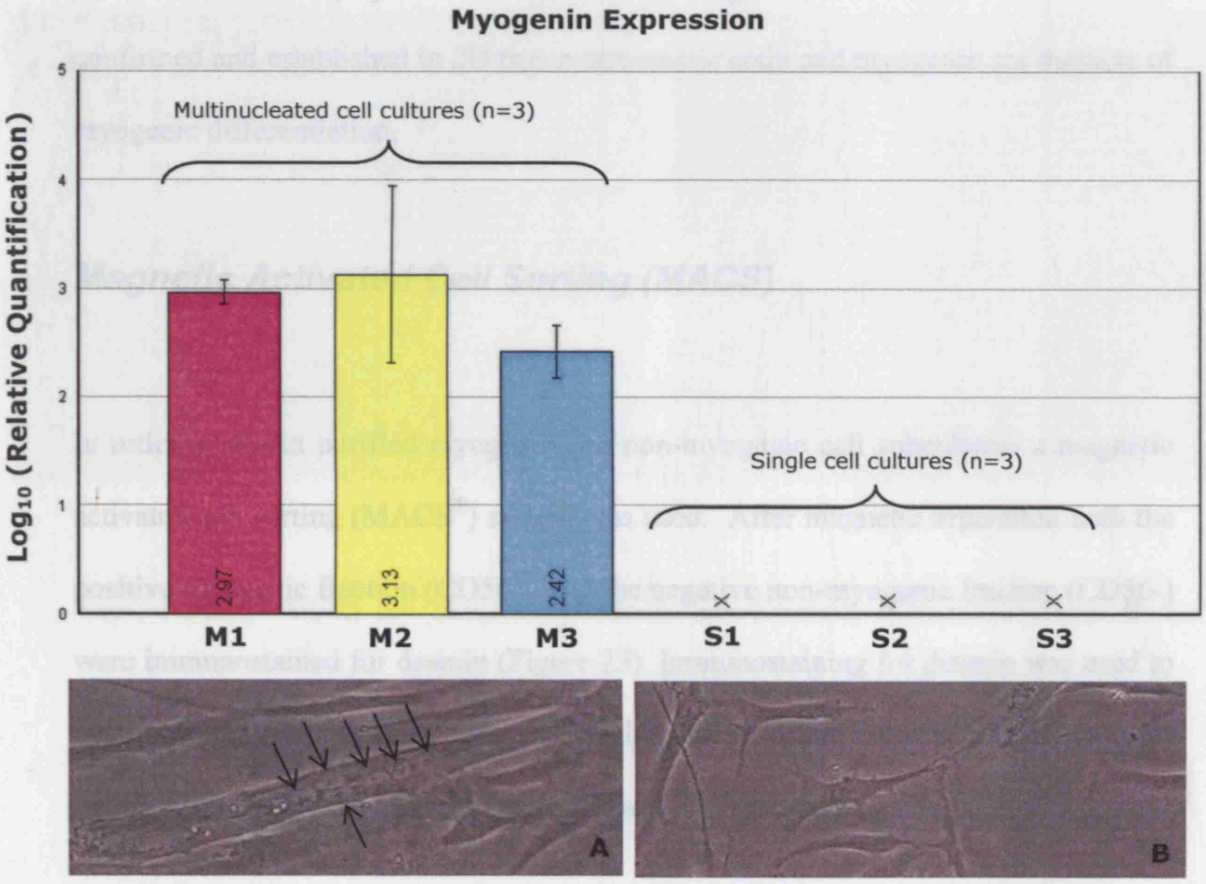

Figure 24: Gene expression plot showing the relative quantification of myogenin expression in multinucleated cell (myotube) cultures (M1-M3; n=3) and single cell (S1-S3; n=3), obtained from the Applied Biosystems® Sequence Detection System (SDS) Software. The '×' indicates no detection of the gene. Error bars represent standard error of the mean expression level calculated from a group of

three replicates. Optical micrographs (×20) of a multinucleated cell culture (A) and a single cell culture (B) are also shown.

RNA was extracted from cultures which contained myotubes visible under the light microscope as described in Materials and Methods. RNA was also extracted from single cell cultures and myogenin expression of both cultures was quantified using RT-PCR. The Applied Biosystems® Sequence Detection System (SDS) Software gene expression plot (Figure 24) shows the expression of myogenin in multinucleated cell cultures, but no myogenin was detected in single cell cultures. These results confirmed and established in 2D that α-sarcomeric actin and myogenin are markers of myogenic differentiation.

Magnetic Activated Cell Sorting (MACS)

In order to obtain purified myogenic and non-myogenic cell subcultures a magnetic activated cell sorting (MACS®) system was used. After magnetic separation both the positive myogenic fraction (CD56+) and the negative non-myogenic fraction (CD56-) were immunostained for desmin (Figure 25). Immunostaining for desmin was used to determine the proportion of myogenic cells present in the respective MDC cultures before (Figure 25A) and after (Figure 25B and 25C) magnetic sorting.

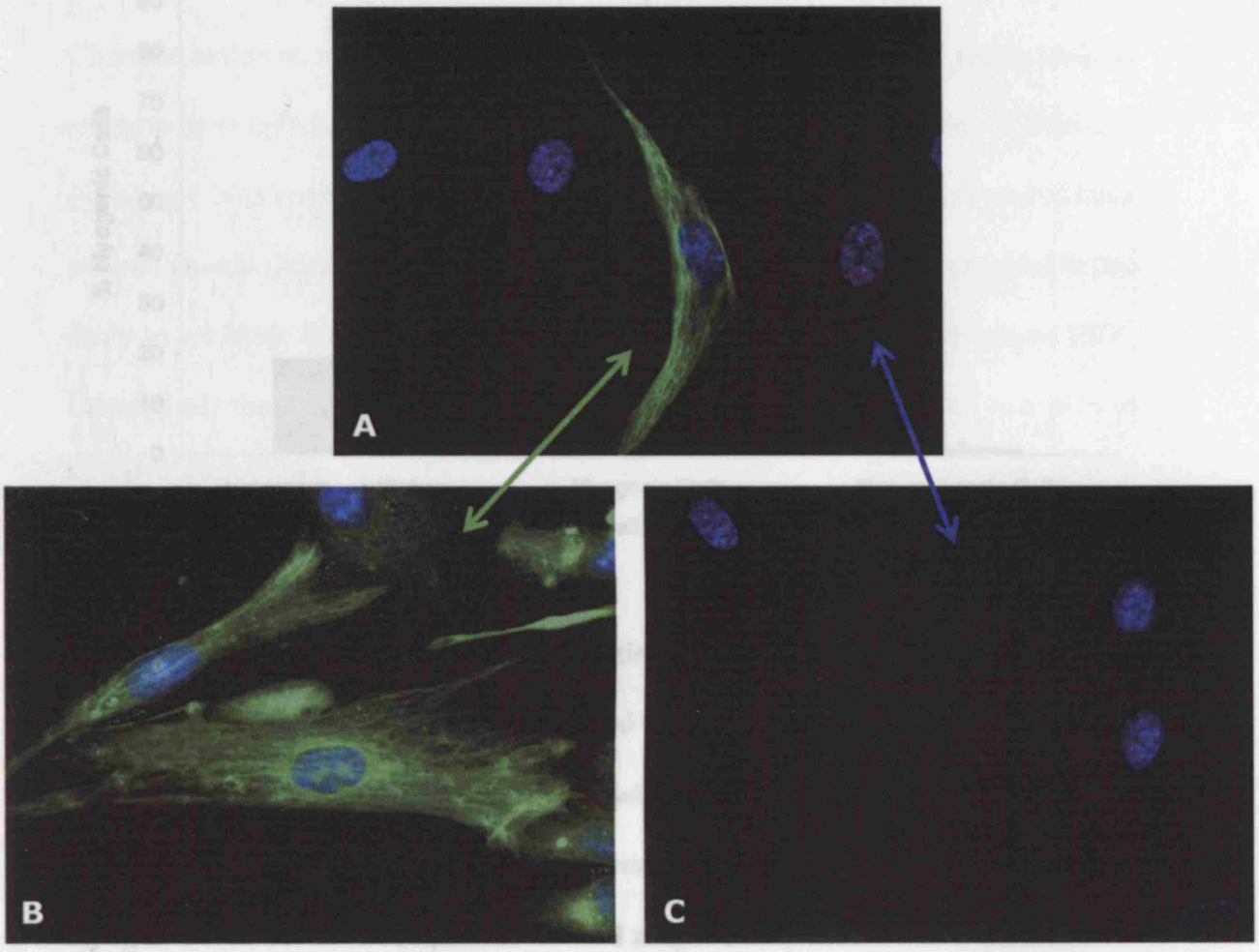

Figure 25: Isolation and purification of myogenic cells from the heterogenous mixture (A) of MDCs was achieved by magnetic cell sorting. These immuno-fluorescent micrographs illustrate desmin expression in cultured human masseter muscle cells before and after separation. The green arrow indicates the purified positive myogenic (CD56+) fraction (B); the blue arrow indicates the negative non-myogenic fraction (C).

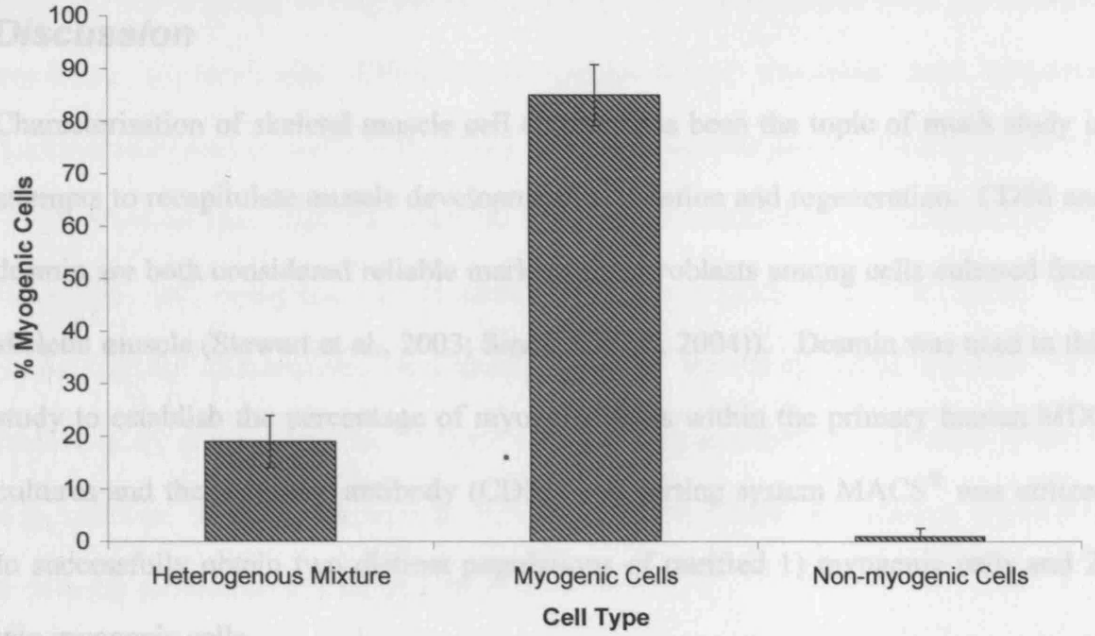

Figure 26: This graph shows the proportion of myogenic cells in the pre-sorted heterogenous mixture of MDCs (n=10+; sd ± 4.9%), the sorted CD56+ myogenic cells (n=10+; sd ±5.8%) and negatively selected non-myogenic cells (n=10+; sd ±1.6%). There was a significant difference ($p<0.0001$) in the percentage of myogenic cells between the pre-sorted and magnetically sorted MDC cultures.

The proportion of myogenic cells (Figure 26) in the pre-sorted heterogenous mixture (19 ± 4.9 %; n=10+) and the two sub-cultures of myogenic (85 ± 5.8%; n = 10+) and non-myogenic (1 ± 1.6%; n=10+) cells were significantly different ($p<0.0001$), this indicated that we now had three distinct cell lineages we could use for further testing: 1) Myogenic cells 2) Non-myogenic cells and 3) the original heterogenous mixture of myogenic and non-myogenic cells.

Discussion

Characterisation of skeletal muscle cell cultures has been the topic of much study in attempts to recapitulate muscle development, adaptation and regeneration. CD56 and desmin are both considered reliable markers for myoblasts among cells cultured from skeletal muscle (Stewart et al., 2003; Sinanan *et al.*, 2004)). Desmin was used in this study to establish the percentage of myogenic cells within the primary human MDC cultures and the magnetic antibody (CD56) cell sorting system MACS® was utilized to successfully obtain two distinct populations of purified 1) myogenic cells and 2) non myogenic cells.

Under phase contrast it was impossible to distinguish myogenic from non-myogenic cells by size or shape (morphology), therefore, immunohistochemical techniques were employed. Antibodies to muscle cell antigens have become increasingly useful for studying the distribution and behaviour of these cells both *in vitro* and *in vivo* (Schultz and McCormick, 1994). When a myogenic cell in culture is stained with an antibody to a cytoplasmic intermediate filament protein such as desmin, a delicate network of threadlike filaments is usually seen surrounding the nucleus and extending through the cytoplasm to the plasma membrane as depicted in Figure 22. Further, when single primary myogenic MDCs reached confluence and standard growth medium was changed to differentiation medium multinucleated myotubes were present in the cultures (within 1 day after the change of medium) and immunostaining for α-sarcomeric actin confirmed terminal differentiation.

Skeletal myogenesis is a highly ordered process within a given cell. Although some aspects of muscle differentiation and cell cycle control have been elucidated, the

temporal relationships among the events that govern the transition from proliferative myoblasts to terminally differentiated multinucleated myotubes have remained controversial (Andrés and Walsh, 1996). Using molecular probes, Andrés and Walsh (1996) showed that after the onset of terminal differentiation, as indicated by the expression of myogenin, a myoblast withdraws from the cell cycle, then phenotypically differentiates, and finally, fuses to form a syncytial myotube. The present study showed that in 2D there was no myogenin mRNA expressed in single cell cultures; however myogenin mRNA was expressed in cultures containing terminally differentiated multinucleated myotubes.

Sinanan et al., (2004) highlighted the importance of obtaining cultures that are as near to 100% myogenic as possible for the study of the molecular and cellular events involved in human myotube formation and to investigate efficiently the interactions between myoblasts and other cells. Using immunomagnetic cell sorting techniques two subcultures of myogenic MDCs and non-myogenic MDCs were obtained. Myogenic purification yields were consistent with those reported by previous researchers (Lequerica *et al.*, 1999; Sinanan *et al.*, 2004). Lequerica et al. (1999) reported an increase in the percentage of myoblasts in mixed culture from 8.4% to more than 90% using this technique. We obtained a purified myoblast subpopulation which expressed desmin in at least 85% of the cells (Figure 26).

Conclusions

- This study has demonstrated that cells derived from the human masseter muscle are a heterogenous mixture of myogenic and non-myogenic cells that are indistinguishable under phase contrast. By using immunohistochemical techniques the percent of myogenic cells of the primary heterogenous mixture of MDCs was determined.

- Under known fusion conditions of confluence and reduced serum in the medium it was confirmed that primary human craniofacial MDCs differentiate to form multinucleated myotubes, verified by immunostaining for α-sarcomeric actin.

- The myogenic regulatory factor myogenin is a marker for muscle cell differentiation being expressed in differentiated multinucleated cell cultures but not expressed in single cell cultures.

In conclusion, using MACS® we successfully obtained and quantified two purified subcultures of myogenic and non-myogenic cells from the heterogenous mixture of primary human MDCs. This study was important, as a necessary pre-requisite for the thesis to follow, establishing the skeletal muscle markers of myogenic cells (desmin) and differentiation (α-sarcomeric actin and myogenin) in 2D which will be used for subsequent 3D culture studies to indicate differentiated myogenic cells (myotubes).

Ch 2: 3D MDC cultures, matrix remodelling and expression of muscle specific genes

Introduction

Understanding aspects of cell behaviour in terms of changing morphology, biochemistry and genetics has all has been made possible by our ability to remove cells from their native environment and culture them *in vitro*. The majority of cell culture work is carried out in 2D, with cells grown on flat, stiff plastic surfaces. These cultures are not only easy to maintain, but easy to visualise and study. With this ease has come a much greater understanding of fundamental muscle cell biology. However, these 2D cultures, though conveniently used for the maintenance of cells and for developmental studies, impose highly unnatural geometric and mechanical constraints on the cells (Beningo et al., 2004). Such models cannot replicate the mechanical responses and signaling contexts that occur when muscle cells are completely surrounded by a matrix that can be remodeled, such as occurs in the 3D native tissue environment (Grinnell, 2003).

It is becoming apparent that the circumstances related to the behaviour of cells in three-dimensional scaffolds *in vitro* are revealing aspects of the phenotypes of cells and insights into cell behaviours that would have otherwise escaped view (Bach *et al.*, 2004). Cytomechanics (cell-level mechanics) characterises the complex interplay of cell forces and the material through which they operate (Brown, 2006). By understanding this interplay of cell-level forces with extracellular matrix material properties novel insights into skeletal muscle adaptation and remodeling are possible,

also this knowledge can be harnessed in applications like developing 3D *in vitro* models for tissue engineering of skeletal muscle.

To study cell cytomechanical physiology, three dimensional (3D) cultures of myoblasts have been developed by a number of groups (Vandenburgh and Karlisch, 1989; Dennis and Kosnik, 2000; Cheema et al., 2003). The pivotal feature for cells in a 3D matrix is that substrate attachment cues act through all 3 axes of the cell, generating completely new directional information, physiologically comparable to that in tissues (Brown and Philips, 2007). Further, use of 3D culture models are central to the hypothesis that tension is crucial for normal muscle development, function, and myoblast differentiation (Cheema et al., 2003).

Collagen I is a structurally important component in the interstitial extracellular matrix of skeletal muscle (Shah et al., 2005). The use of collagen as a 3D matrix support is consistent with its role in normal muscle (Cheema et al., 2005). In this study primary human MDCs were seeded in a 3D collagen type I construct. The objective was to quantitate mechanical remodelling of a 3D matrix by MDCs on the culture force monitor (CFM; Eastwood et al., 1994, 1996) using isometric conditions (i.e. attached which is closer to the physiological setup than a free floating matrix).

Using the CFM, previous workers have established that contractile forces are primarily generated as cells attach to the collagen fibrils within the 3D construct (Eastwood *et al.*, 1994; Brown *et al.*, 1998; Mudera *et al.*, 2000; Cheema *et al.*, 2003; Karamichos *et al.*, 2006). The peak force depends on cell type, attachment, density,

growth factors and matrix stiffness (Karamichos, 2006). Many cell types generate a characteristic force contraction profile described by three distinct phases (Figure 27):

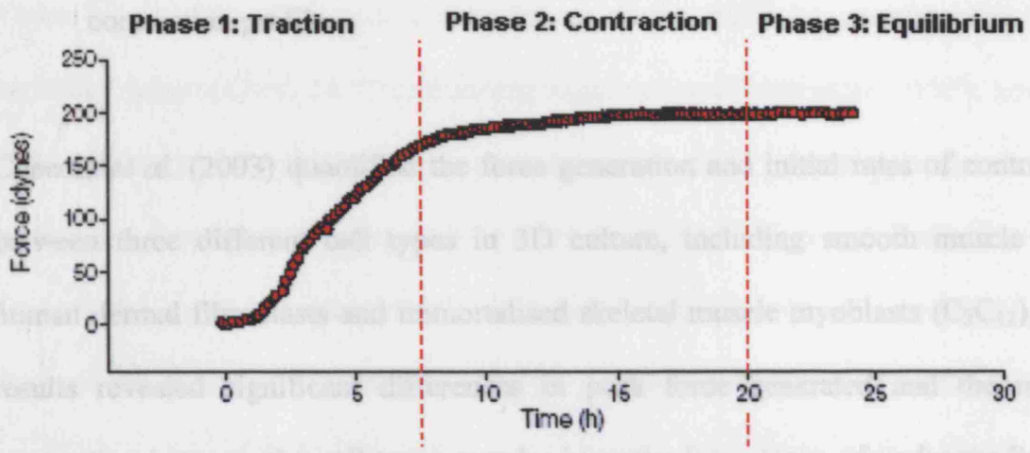

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Figure 27: The CFM allows force measurement of cell populations in uniaxially tethered collagen lattices. This characteristic force-time plot indicates three distinct phases of cell force generation. In phase 1 the cells exert tractional forces on the collagen matrix as they attach. In phase 2 the cells generate contractile force that is maintained relatively constant. Phase 3 is when equilibrium (tensional homeostasis) is reached (adapted from Tomasek *et al.*, 2002).

1. **Traction (0-8hrs):** initial force generation is thought to be due to traction as cells attach and spread through the collagen constructs (Tomasek *et al*, 2002). Understanding this traction phase is important since it both facilitates and perhaps predicts the level of force generation and matrix remodelling (Tomasek *et al*, 2002).
2. **Contraction (8-20hrs):** a linear increase in force generation is observed as cells continued to remodel the matrix and

3. **Equilibrium (20-24hrs):** or tensional homeostasis is attained when the cells have established an equilibrium tension within their extracellular collagen matrices. This is evidenced by a characteristic 'plateau' on the force contraction profile.

Cheema *et al.* (2003) quantified the force generation and initial rates of contraction between three different cell types in 3D culture, including smooth muscle cells, human dermal fibroblasts and immortalised skeletal muscle myoblasts (C_2C_{12}). The results revealed significant differences in peak force generated and the rate of contraction between the cell types, emphasising the importance of understanding the behaviour of single cell types in cytomechanical studies before using mixed cell populations or co-cultures, like primary muscle cultures (Cheema *et al.*, 2003). It is presently uncertain whether the forces generated by the heterogenous mixture of myogenic and non-myogenic cells are simply additive or if they act synergistically (i.e. combined effect is greater than sum of their individual effects) (Cheema, 2004). *The first aim of this study was to quantitate the forces generated by the heterogenous mixture of myogenic and non-myogenic primary human craniofacial MDCs embedded within the 3D construct (collagen) as these cells remodelled the matrix over a period of 24 hours. To test the hypothesis that the forces generated by isolated myogenic (CD56+) and non-myogenic cells during remodeling were cumulative (as opposed to synergistic).*

Remodeling of skeletal muscle connective tissue is required as part of that tissues adaptive response and it is known that the MMP family of enzymes is required for such tissue remodeling (Auluck et al., 2005). MMPs play an essential role in the

maintenance of surrounding connective tissue by regulating the integrity and composition of the ECM in skeletal muscle (Matrisian, 1992; Carmeli et al., 2004).

There is evidence of extensive remodeling of the ECM by muscle cells during myoblast fusion (Chen, 1977) and during regeneration (Gulati et al., 1983); however, little is known of the ability of myoblasts to secrete ECM-degrading enzymes and to participate directly in the remodelling of their ECM (Guérin and Holland , 1995). One of the most important MMPs associated with skeletal muscle function and dysfunction appears to be MMP2, a known mechanoresponsive gene that has been used as a marker of ECM remodeling (Mudera et al., 2000; Auluck et al, 2005). It has been suggested that both myogenic and non-myogenic cells participate directly in remodeling of the ECM during myogenesis and the regeneration of skeletal muscle (Tippett, 1997). However, the relative contribution of these two cellular components to the overall MMP activity in muscle is unknown (Singh et al., 2000; Lewis et al., 2001). The activity of the MMPs at both mRNA and/or protein levels modulates the degradation of the ECM components (Corcoran et al., 1996; Lluri and Jaworski, 2005). *The second aim of this study was to quantitate the expression of MMP2 mRNA as a marker of matrix remodeling in 3D cultures of primary human MDCs and establish the relative contribution of the isolated subpopulations of myogenic and non-myogenic cells. Testing the hypothesis that both myogenic and non-myogenic cells contribute to the regulation of MMP2 gene expression.*

Skeletal muscle differentiation entails the coordination of muscle-specific gene expression and terminal withdrawal from the cell cycle (Halevy *et al.*, 1995). Myogenin is required for formation of differentiated muscle fibers and activates a

broad array of muscle specific genes in cultured cells (Hasty *et al.*, 1993). Andrés and Walsh (1996) suggested an overall order of marker expression (Figure 28) during *in vitro* myogenesis:

1. Entry of myoblasts into the differentiation pathway, as indicated by the induction of **myogenin; and.**
2. Phenotypic differentiation, as indicated by the induction of myosin heavy chain (**MyHC**).

However, they do not rule out that these processes are stochastic at the level of the individual cell (Andrés and Walsh, 1996). Myogenin and MyHC are established markers of differentiation in 2D (Hasty *et al.*, 1993; Halevy *et al.*, 1995; Andrés and Walsh, 1996) and expression of these genes will be monitored in 3D MDC cultures in the studies to follow as a quantitative measure of myogenic cell differentiation.

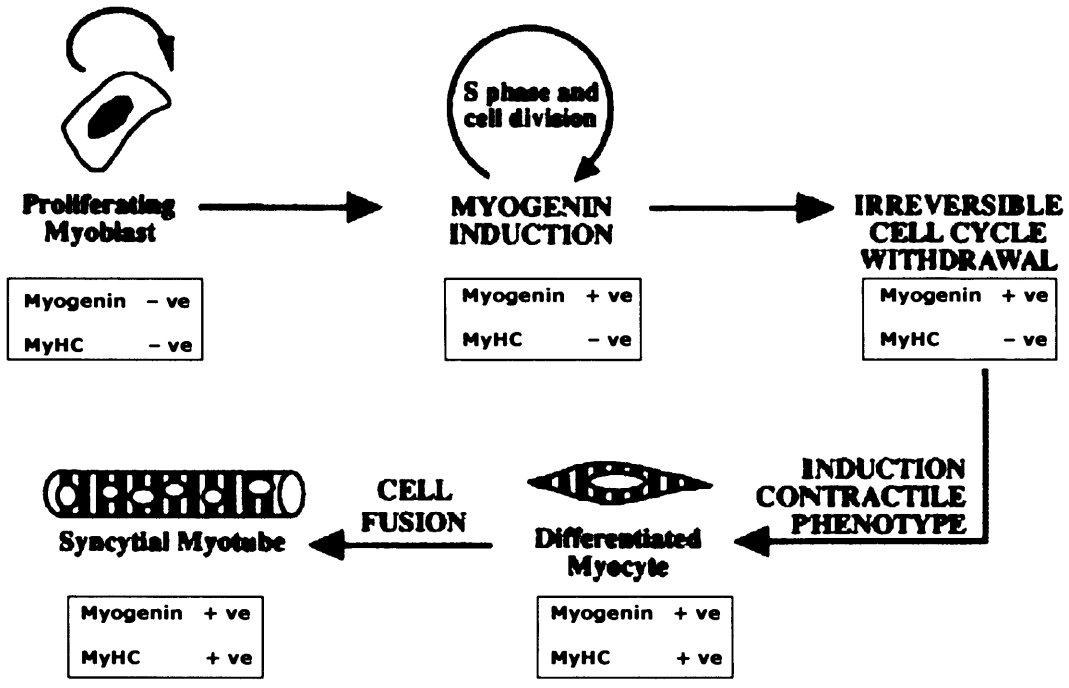


Figure 28: Marker expression during 2D *in vitro* myogenesis (adapted from Andrés and Walsh, 1996). Proliferating myoblasts do not express myogenin or

MyHC. Upon terminal differentiation myogenin is expressed. MyHC is induced in the differentiated myocyte and determines the contractile phenotype of the resulting syncytial myotube which expresses both myogenin and MyHC.

As stated in chapter 1, there are no reports in literature that have investigated the interactions between muscle fibres, myogenic cells, non-myogenic cells and the ECM (Tippett, 1997; Lewis et al., 2000, Singh et al., 2000). Tippett (1997) suggested that the nature and level of interactions between the cellular components and their environment may well determine the expression of muscle specific proteins (e.g. myogenin, MyHC). *The final aim of this study was to quantify the molecular output (muscle specific gene expression i.e. myogenin, MyHC-β and MyHC IIX/D) of the heterogenous mixture and the separated myogenic cellular component of MDCs seeded in a 3D collagen gel using relative quantitative RT-PCR. To test the hypothesis that after 24 hours in 3D culture isolated myogenic cells express significantly increased levels of muscle specific mRNA in 3D culture than the heterogenous mixture containing non-myogenic cells.*

Materials and methods

Briefly, the heterogenous mixture of primary human MDCs and the subcultures of purified myogenic and non-myogenic cells were seeded in collagen type I constructs as previously described. They were then tethered to the CFM for 24 hours to monitor matrix remodeling and the molecular output was quantified using RT-PCR as previously described.

Results

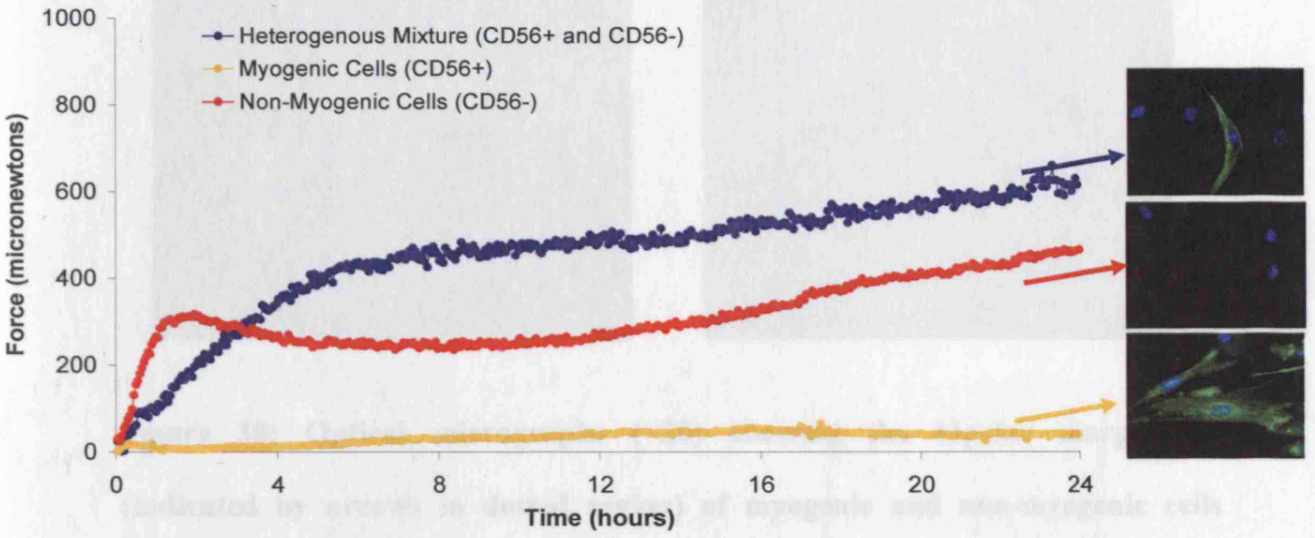


Figure 29: Comparison of the force contraction profiles obtained as the three types of MDCs remodelled a 3D extracellular matrix. The contraction profiles were obtained by seeding 5 million cells into a 5mL collagen gel (n=3). Immediately following gelling, the contraction of the collagen matrix by the resident cells was measured using the CFM instrument over a 24 hour period.

Matrix remodeling was quantified by measuring the force generated (Figure 29) as primary human MDCs attached to (Figure 30) and remodeled the 3D collagen gel. Two significant cytomechanical properties were established; firstly, the peak force generated over 24 hours and secondly, the initial rate of force generation. The initial rate of force generation was calculated as the change in force over time from 0-60 minutes of monitoring. The non-myogenic cells had the highest initial rate of contraction (Figure 32; 4.4 ± 1.69μN/min; n = 3), though, this cell type did not generate the corresponding highest peak force (Figure 31; 465 ± 56μN; n = 3).

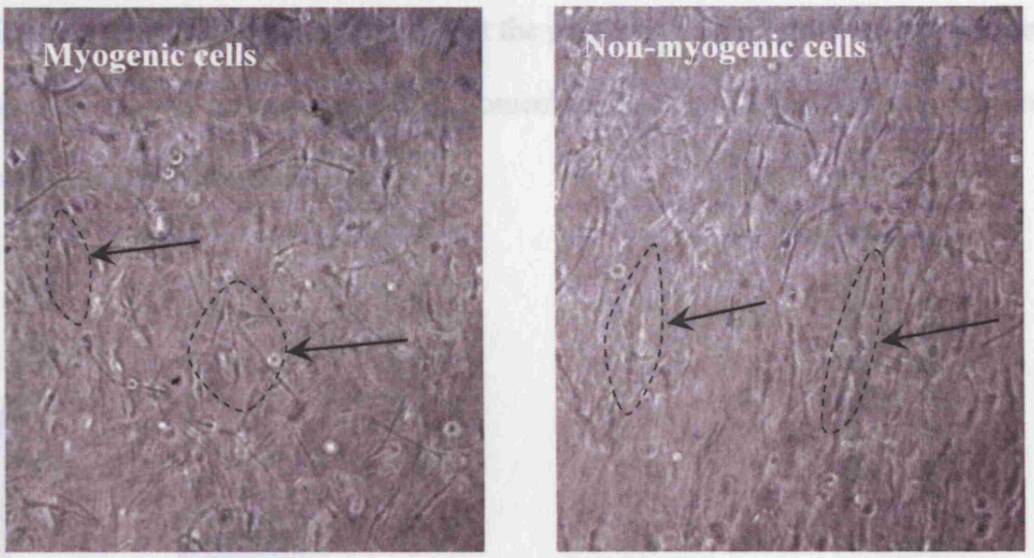


Figure 30: Optical micrographs (×20) showing the bipolar morphology (indicated by arrows in dotted region) of myogenic and non-myogenic cells attached (evidenced by the extended cell processes) to the 3D collagen matrix after 24 hours.

The myogenic cells were classified as low contractile (Karamichos, 2006) as they generated little to no measurable force in the first hour at a rate of (0.1 ± 0.31μN/min) reaching a peak force of 40 ± 50μN (Figure 31; n = 3) over 24 hours. The heterogenous mixture of MDCs embedded in the collagen matrix generated the highest peak force (Figure 31; 615 ± 155μN; n = 4), with an initial rate of force generation being 1.6 ± 0.34μN/min (Figure 32; n = 4). The peak force attained by the heterogenous mixture was greater than the sum (505μN) of the individual myogenic and non-myogenic sub-populations, suggesting force synergism. In summary, the typical mean force generation profiles generated by the three cell types (Figure 29) studied showed significant differences in peak force attained ($p<0.01$; Figure 31) and the initial contraction rate (evaluated between 0 and 60 minutes) ($p<0.001$; Figure 32). This is the first cytomechanical analysis of primary human MDCs embedded in a

3D matrix and these results indicate that the proportion of myogenic to non-myogenic cells within a 3D matrix affects the cytomechanical response (i.e. rate and peak force generation) of the cultures.

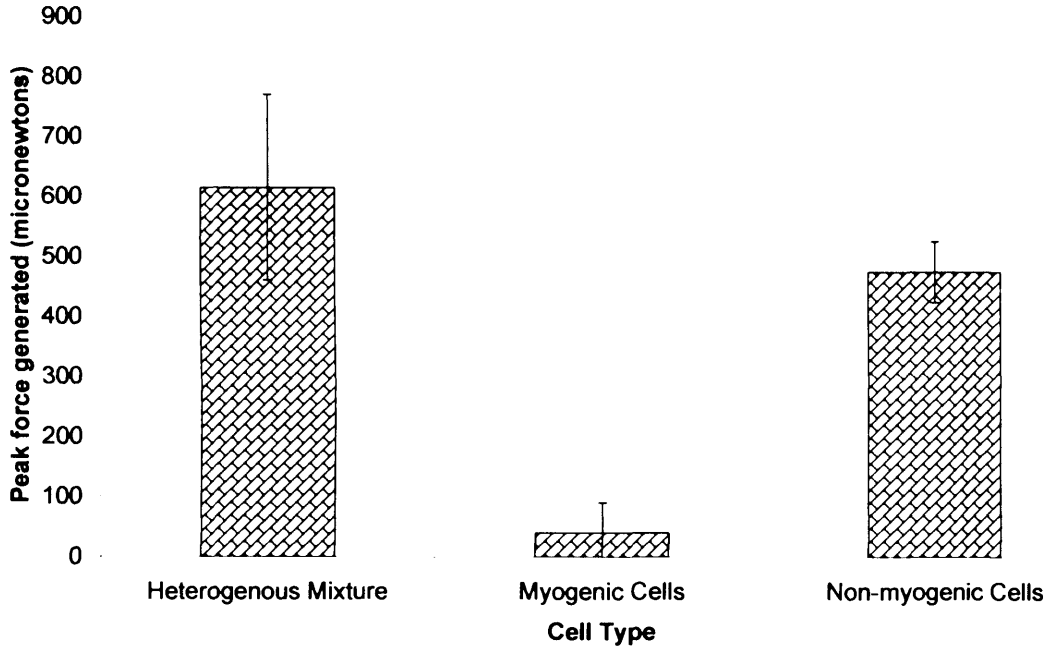


Figure 31: Graph showing the peak force generated by the three primary human craniofacial MDC types. There were significant differences ($p<0.01$) between the peak force attained for the heterogenous mixture (n = 4; sd ± 155μN), myogenic cells (n = 3; sd ± 50μN) and non-myogenic cells (n = 3; sd ± 56μN).

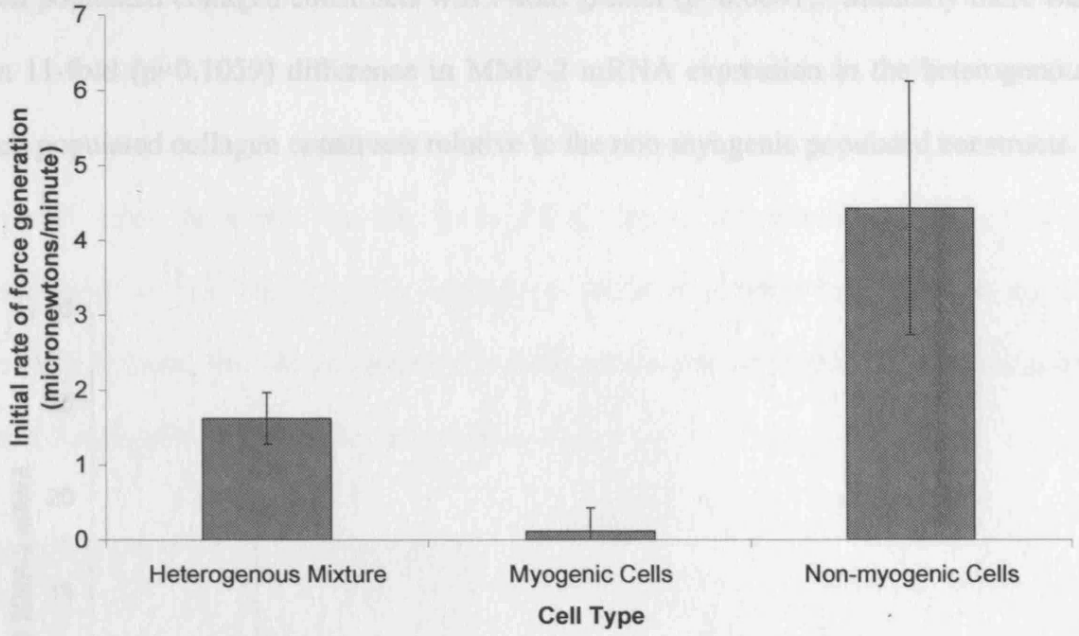


Figure 32: Comparison of mean initial rates of contraction from between 0-60 minutes (i.e. on an initial time basis) for the three cells types. There were significant differences ($p<0.001$) between the rate at which the heterogenous mixture (n = 4; sd ± 0.34μN/min), the myogenic cells (n = 3; sd ± 0.31μN/min) and the non-myogenic cells (n = 3; sd ± 1.69μN/min) contracted the 3D collagen gel.

To further investigate and quantitate remodelling of a 3D collagen matrix by primary human MDCs, MMP2 mRNA expression of these cultures after 24 hours was quantified. As previously stated, MMP2 is used as a marker of ECM remodelling (Mudera et al., 2000; Auluck et al., 2005). The relative contribution of MMP-2 mRNA expression by myogenic and non-myogenic cells to overall expression of MMP2 m-RNA by the heterogenous mixture in 3D MDC constructs was evaluated using RT-PCR. After 24 hours in 3D constructs the relative MMP-2 mRNA expression of each cell type was determined (Figure 33). Relative to the non-myogenic cell populated collagen constructs, MMP-2 mRNA level in the myogenic

cell populated collagen constructs was 7-fold greater (p=0.0641). Similarly there was an 11-fold (p=0.1059) difference in MMP-2 mRNA expression in the heterogenous cell populated collagen constructs relative to the non-myogenic populated constructs.

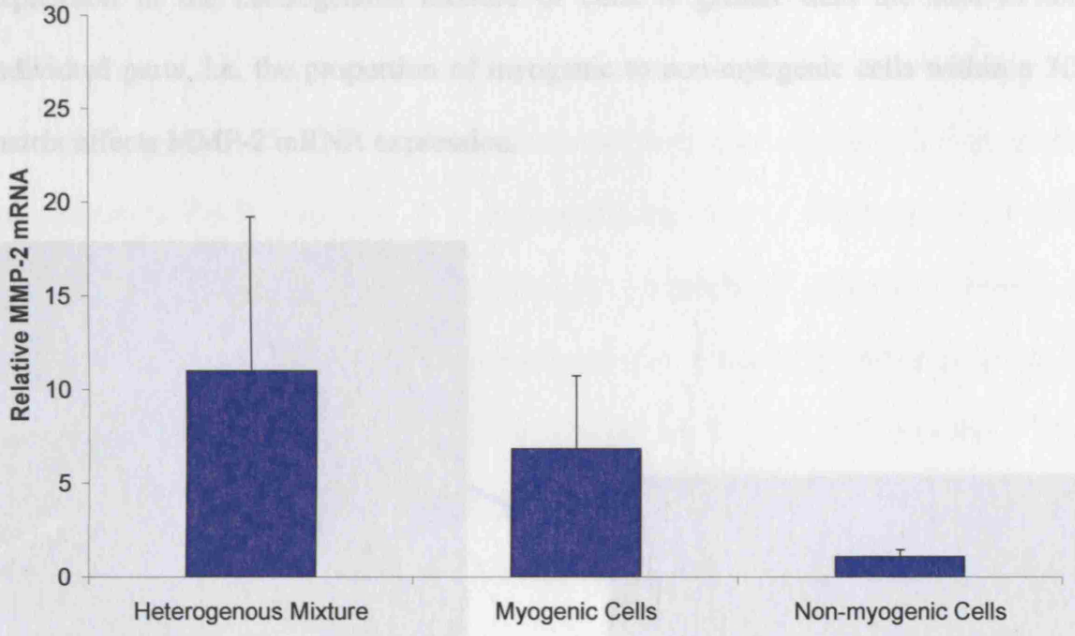


Figure 33: Graph depicting the relative contribution of MMP-2 mRNA expression by myogenic and non-myogenic cells to overall expression of MMP2 m-RNA by the heterogenous mixture in 3D MDC cultures. Relative mRNA expression levels were determined by RT-PCR. Values representing the amounts of MMP2 mRNA were relative to GAPDH. Error bars represent standard deviation (Heterogenous Mixture ± 8.2; Myogenic cells ± 3.9; Non-myogenic cells ± 0.4) and by ANOVA statistical analysis these results were approaching significance (p=0.06).

These results clearly indicate that both myogenic and non-myogenic cells contribute to the overall MMP-2 produced in constructs, with the myogenic cells expressing relatively more MMP2 mRNA than their non-myogenic counterpart. Further, as with overall force generated by the three MDC types, the overall MMP-2 mRNA expression in the heterogenous mixture of cells is greater than the sum of the individual parts, i.e. the proportion of myogenic to non-myogenic cells within a 3D matrix affects MMP-2 mRNA expression.

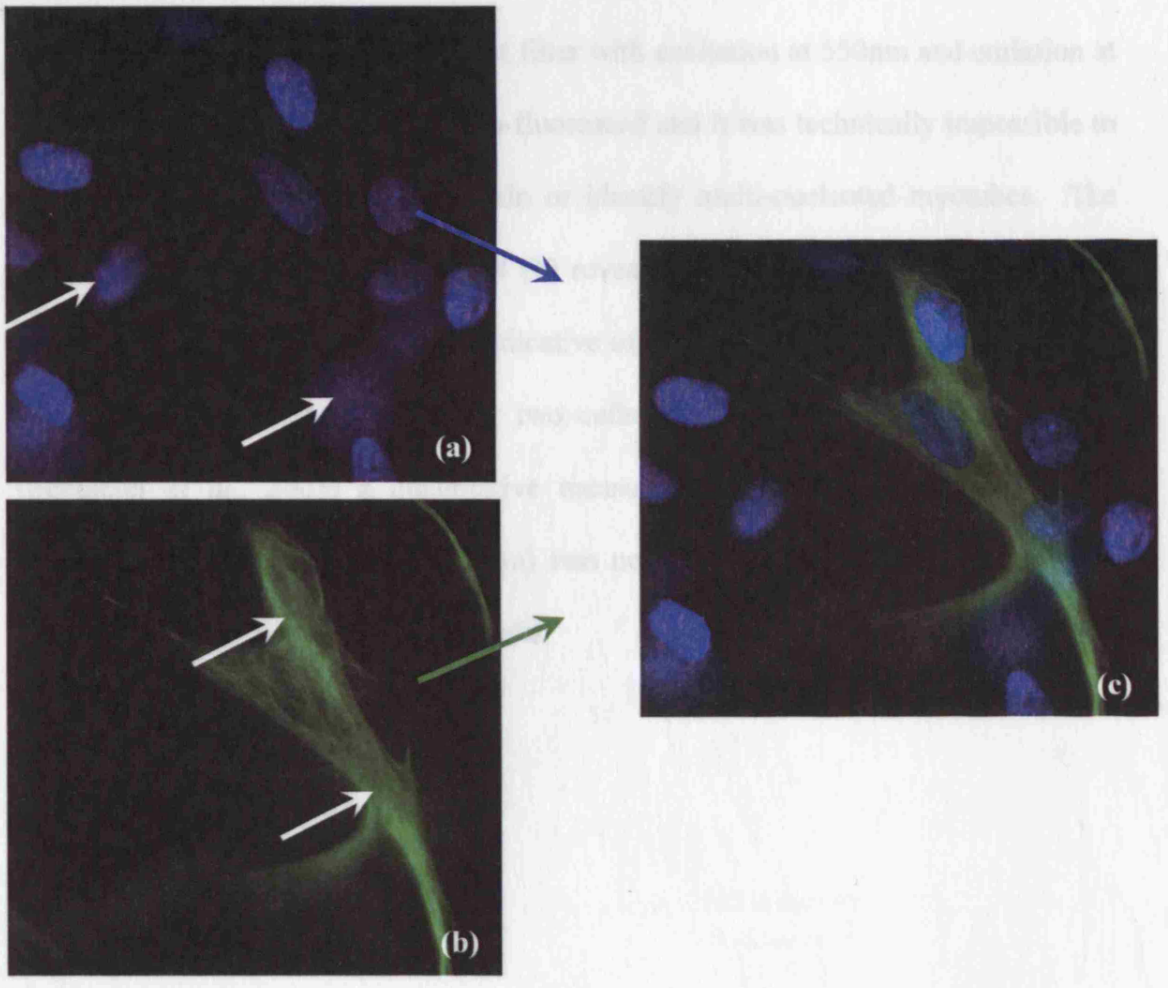


Figure 34: MDCs seeded at 5 million/5ml collagen gel immunostained (magnification 63×) with DAPI (blue) and Desmin (green). The white arrows (a) indicate the 'blurred' nuclei which belonged to different planes of the 3D gel.

The white arrows (b) indicate desmin filaments of myogenic cells within the gel. The blue and green arrows point to the composite image of (a) and (b) depicting the close proximity and possible fusion of two myoblasts within the 3D construct.

The MDC seeded 3D collagen gels were immunostained for desmin (myogenic marker; Figure 34) and α-sarcomeric actin (marker of terminal differentiation) to identify myogenic cells and fused multinucleated myotubes respectively, within the 3D constructs. DAPI was used as a counterstain to identify the nuclei of all cells (Figure 34 (a)). Under the fluorescent filter with excitation at 550nm and emission at 570nm the hydrated collagen gel auto-fluoresced and it was technically impossible to image the red α-sarcomeric actin stain or identify multi-nucleated myotubes. The desmin filaments shown in Figure 34 (b) reveal two nuclei in close proximity with overlapping intermediate filaments indicative of fusion; however, because there is no way of clearly ascertaining whether two cells are truly fused or closely apposed (Schuierer *et al.*, 2005) a quantitative measure of myogenic differentiation (i.e. myogenin and MyHC gene expression) was necessary to confirm differentiation of myogenic cells in 3D collagen constructs.

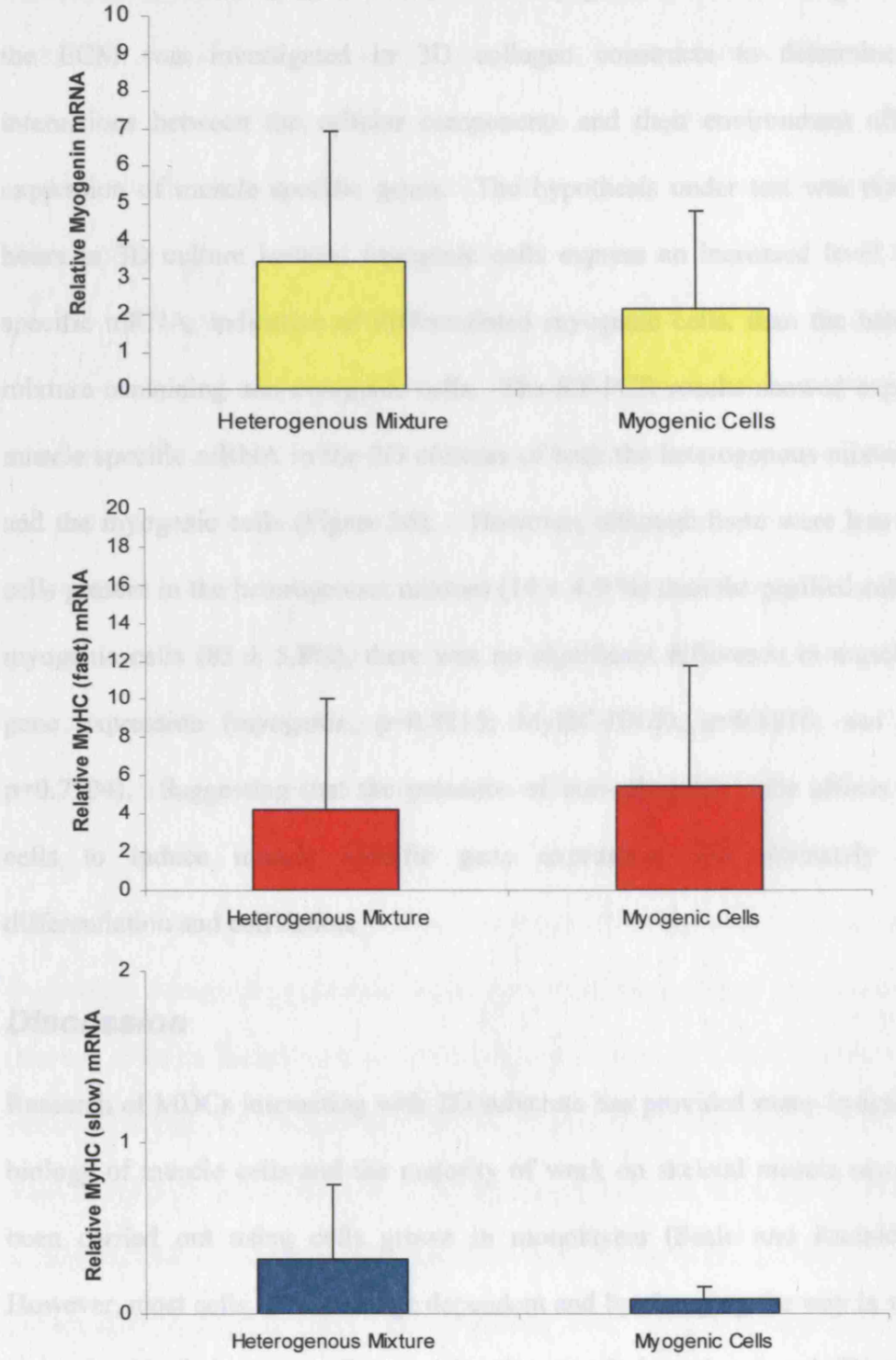


Figure 35: Relative muscle specific (Myogenin and MyHC (fast and slow)) gene expression is shown for the heterogenous mixture and myogenic cells when seeded at a density of 5 million/5ml collagen gel (n=3). There was no significant difference in gene expression. Standard deviation is used for error bars.

The nature and level of interaction between myogenic cells, non-myogenic cells and the ECM was investigated in 3D collagen constructs to determine whether interactions between the cellular components and their environment affected the expression of muscle specific genes. The hypothesis under test was that after 24 hours in 3D culture isolated myogenic cells express an increased level of muscle specific mRNA, indicative of differentiated myogenic cells, than the heterogenous mixture containing non-myogenic cells. The RT-PCR results showed expression of muscle specific mRNA in the 3D cultures of both the heterogenous mixture of cells and the myogenic cells (Figure 35). However, although there were less myogenic cells present in the heterogenous mixture (19 ± 4.9 %) than the purified subculture of myogenic cells (85 ± 5.8%), there was no significant difference in muscle specific gene expression (myogenin, $p=0.8215$; MyHC-IIX/D, $p=0.8815$; and MyHC-β, $p=0.7304$). Suggesting that the presence of non-myogenic cells affects myogenic cells to induce muscle specific gene expression and ultimately myogenic differentiation and cell fusion.

Discussion

Research of MDCs interacting with 2D substrata has provided many insights into the biology of muscle cells and the majority of work on skeletal muscle myoblasts has been carried out using cells grown in monolayers (Seale and Rudnicki, 2000). However, most cells are anchorage dependent and by changing the way in which cells interact with their surrounding matrix, the morphology is altered (Discher *et al.*, 2005). 2D monolayer cultures always provide polarised directional cues with a 'dorsal' fluid covered surface and a 'ventral' plastic attached surface (Brown and Phillips, 2007). Conventional 2D monolayer culture systems recreate only a portion of the cellular environment and there is increasing interest in developing more

physiologically relevant 3D culture systems (Stegemann *et al.*, 2005). Cultured within a 3D extracellular matrix such as collagen, primary human MDCs experience a richer, more complex physical environment and morphology results displayed a markedly different geometry in 3D (stellate/bipolar) than cells on 2D surfaces (flattened/spread).

The function and the maintenance of tissue integrity are dependent on specific interactions of cells with the surrounding extracellular matrix (Mayer, 2003). Cell-cell and cell-matrix interactions are of vital importance not only for proper cellular homeostasis during embryogenesis and development of an organism, but also in growth, regeneration, adaptation and pathological states (Kaushal and Shah, 2000). By culturing cells in a 3D matrix the geometry of the integrins and focal adhesions on the cell surface can be mimicked (Grinnell *et al.*, 1999).

The special feature of biological materials is that the resident cells are not passive, but themselves change the material properties in a manner unique to biomaterial science (Brown, 2006). Discher *et al.* (2005) reported how cells feel and respond to the stiffness of their substrate stating that the feedback of local matrix stiffness on cell state has important implications for development, differentiation and regeneration. In a standard 2D culture the only dynamic factor is the cells, as the plastic is non-deformable. Culturing cells in compliant 3D collagen matrices offers new opportunities to understand the reciprocal and adaptive interactions that occur between the cells and surrounding matrix in a tissue-like environment (Grinnell, 2003).

Collagen is a flexible but inextensible fibrillar structure with a stiffness of 30-100Pa as measured by a dynamic mechanical analyzer (Wang *et al.*, 2003) and lies far lower than that of a normal cell culture dish which is not flexible and with a stiffness of more than 1GPa (Wang *et al.*, 2003). During cell–collagen matrix remodeling, mechanical signals from the remodeled matrix feed back to modulate cell shape and behavior (Tomasek *et al.*, 2002). The traction exerted by cells during their migration induces not only translocation of the cells, but also remodeling of the matrix, as the forces generated move both cells and collagen fibrils as depicted in Figure 36.

There is however, the added complexity of the inherent process of myogenic cell differentiation, for which cell–cell attachment is paramount in muscle derived cultures. This presents another mode by which the cells may contract the matrix. In summary, there are two proposed modes by which muscle derived cells could generate force within a 3D collagen lattice:

1. Directly: in which cells attach directly to fibrils of the collagen matrix. When these cells apply tensile forces to the attachment points the collagen lattice itself deforms locally (as depicted in Figure 36; Tomasek et al., 2002).
2. Indirectly: the other possible mechanism of force generation by myogenic cells is the transfer of cell-force indirectly. It has been postulated that skeletal myoblasts have a greater tendency to form cell-cell attachments and will deform the collagen lattice indirectly as a consequence of the contraction of groups of cells (Cheema *et al.*, 2003; Brown, 2006)

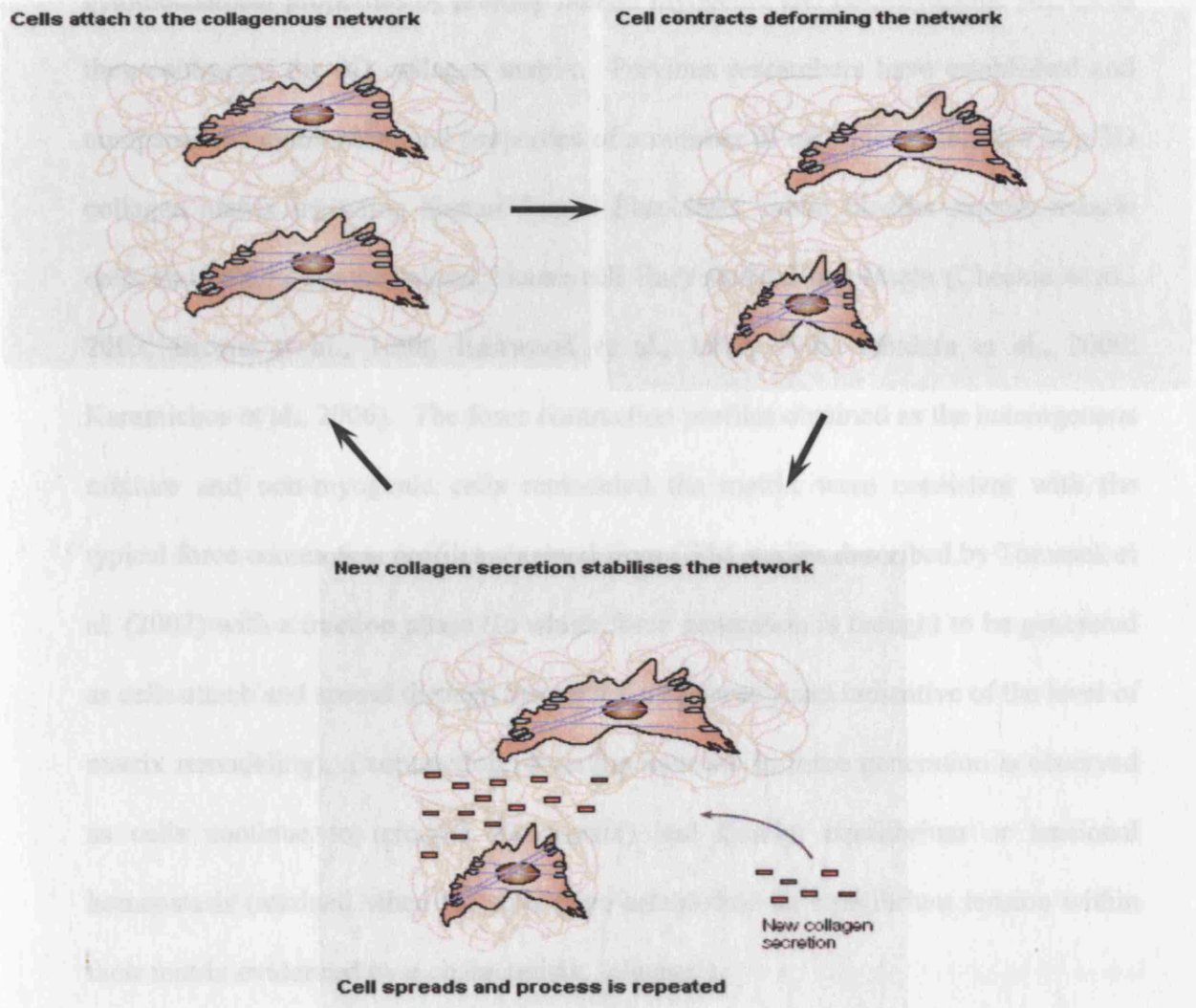


Figure 36: Model of extracellular matrix remodelling mediated by cells embedded in a collagen matrix. In a 3D culture, not only can the surrounding environment affect the cell phenotype, but the cells can also affect their surrounding environment. Cells bind to collagen fibrils which results in local matrix contraction. New matrix components are added to stabilise the new collagen organisation. This process is repeated and small incremental collagen-matrix remodelling occurs (Adapted from Tomasek et al., 2002).

The current study used the CFM to quantify matrix remodeling by monitoring the cytomechanical properties of primary human myogenic and non-myogenic MDCs as they contracted the 3D collagen matrix. Previous researchers have established and compared the cytomechanical properties of a number of cell lines embedded in a 3D collagen matrix including human dermal fibroblasts, rabbit bladder smooth muscle cells and C2C12 (immortalised mouse cell line) skeletal myoblasts (Cheema et al., 2003; Brown et al., 1998; Eastwood et al., 1996, 1998; Mudera et al., 2000; Karamichos et al., 2006). The force contraction profiles obtained as the heterogenous mixture and non-myogenic cells remodeled the matrix were consistent with the typical force contraction profiles obtained from CFM studies described by Tomasek et al. (2002) with a traction phase (in which force generation is thought to be generated as cells attach and spread through the collagen constructs and indicative of the level of matrix remodeling), a contraction phase (an increase in force generation is observed as cells continue to remodel the matrix) and finally, equilibrium or tensional homeostasis (attained when the cells have established an equilibrium tension within their matrix evidenced by a characteristic 'plateau').

As the non-myogenic cells remodelled the 3D collagen gel, most of the force was generated quite rapidly during the traction phase and the initial rate of force generation was significantly higher than the corresponding initial rate of force generation by the heterogenous mixture of cells, which contracted the matrix at a slower, steadier state, but reached a higher peak force after 24 hours in culture. From this we can conclude that the presence of myogenic cells affects the cytomechanical response of non-myogenic cells and vice-versa. There is cell-cell communication resulting in force synergism, i.e., a greater force being generated by the heterogenous

mixture than the sum of the individual parts. The results obtained indicate that the proportion of myogenic cells in the heterogenous mixture significantly affects matrix remodeling.

Enigmatically our results showed that the purified primary human myogenic cells generated little to no measurable force over the 24 hours of monitoring. Interestingly, Cheema *et al.* (2003) noted a delay in force generation or a 'lag phase' in C2C12 myoblast cultures, suggesting that this lag phase is consistent with the idea that C2C12 cells may generate force primarily though cell-cell contact. Cellular attachment to the extracellular matrix results in clustering of integrins and assembly of multi-molecular focal complexes associated with the actin cytoskeleton (Hotchin and Hall, 1995; Cukierman *et al.*, 2001). It has been widely proposed that integrin-matrix interactions influence cell behavior by activating intracellular signal transduction events (Burridge et al., 1988; Juliano and Haskill, 1993; Clark and Brugge, 1995). Myoblast integrin expression is known to change between the differentiated and undifferentiated state (Wakelam, 1985). Morphology after 24 hours in culture revealed that the myogenic cells were spread and appeared to be attached to the matrix; however, a relatively poor cell-matrix attachment might explain why overall contraction was low.

The complex interaction between myogenic and non-myogenic cells derived from skeletal muscle has been the subject of much research over the past two decades. The working hypothesis of Turner (1986) was, *in vivo*, myogenic cells attach to a fibronectin containing extracellular matrix laid down by fibroblasts (non-myogenic), and that this interaction contributes significantly to the organization of muscle. More

recently, it was proposed that the process of skeletal muscle regeneration is influenced by the composition of the extracellular matrix and the availability of growth factors produced by non-myogenic cells (Baj et al., 2005). Another reason why little to no force was generated as the myogenic cells attached to the 3D matrix might be due to the absence of ECM components and growth factors secreted by the non-myogenic cells.

Tissues owe their dynamic structure both to changes in expression of adhesive proteins and their receptors and to the regulated action of secreted proteinases, particularly members of the metalloproteinase family (Kaushal and Shah, 2000). MMP expression is regulated at the transcriptional level and expression is induced by cytokines, growth factors, chemical agents, and cell-cell or cell-matrix interaction (Lluri and Jaworski, 2005). To date, the relative contribution of the two cellular components of muscle to the overall MMP activity in muscle is unknown (Lewis et al., 2001). In the present study to further quantify matrix remodeling and determine the relative contribution of the two cellular components, we monitored the expression of MMP-2 mRNA in 3D cultures of isolated myogenic, non-myogenic and the heterogenous mixture. The results established that the two cellular components express MMP-2 mRNA and therefore both contribute to the overall MMP activity in muscle. Further, the results indicated that myogenic cells encode for the secretion of more of the enzyme than their non-myogenic counterpart, as at the transcriptional level MMP-2 expression was greater in 3D myogenic cell cultures than in 3D non-myogenic cell cultures. There was also relatively increased MMP-2 mRNA in the heterogenous mixture than the sum of the individual components again suggesting

that cell-cell interaction results in greater remodeling of the matrix than the isolated response of the individual components.

Finally, this study reported for the first time the gene expression of myogenin and two MyHC isoforms (fast (MyHC-IIX/D) and slow (MyHC-β)) after 24 hours in 3D culture, indicating the presence of differentiated myogenic cells within the constructs. The fact that there was no significant difference between the muscle specific gene expression of markers of myogenic differentiation in the heterogenous mixture of cells and the myogenic cells is consistent with the finding of Cooper *et al.* (2003) in 2D who suggested that the presence of non myogenic cells enhances differentiation of myogenic cells. Although there were less myogenic cells in the heterogenous mixture, factors secreted by the non-myogenic cells stimulated the differentiation of a similar proportion of myogenic cells as occurred in the purified myogenic cell populated 3D collagen gel.

Conclusion

- Using the CFM, this study characterised for the first time, the forces generated as primary human MDCs remodelled 3D collagen gel. The cytomechanical properties of the heterogenous mixture, and the separated cellular (myogenic and non-myogenic) components, revealed that both the rate of force generation and the peak force generated were not cumulative, but synergistic. Concluding that the proportion of myogenic to non-myogenic cells within the 3D culture affects the cytomechanical response.

- MMP2 mRNA was monitored as a marker of matrix remodeling and this study established that both the myogenic and non-myogenic cellular components contribute to the production of MMP2 in 3D culture. Further, the overall MMP-2 mRNA expression in the heterogenous mixture of cells was greater than the sum of the individual parts, concluding that the proportion of myogenic to non-myogenic cells within a 3D matrix affects MMP-2 mRNA expression.

- This study has established that myogenic cells do not express a significantly increased level of muscle specific mRNA in 3D culture than the heterogenous mixture containing non-myogenic cells. Concluding that the interactions between the cellular components and their environment determine gene expression of muscle specific markers (myogenin and MyHC) of differentiation.

In conclusion, the objective to use 3D MDC cultures, using primary cells from adult human craniofacial masseter muscle, to investigate and quantify skeletal muscle remodeling and myogenic cell differentiation in 3D culture was achieved. From this study we conclude that the two cellular components of muscle contribute to skeletal muscle remodeling and gene expression of markers of myogenic differentiation in 3D cultures. Future studies to tissue engineer a 3D craniofacial muscle organoid should utilize the heterogenous mixture of myogenic and non-myogenic cells as found in the native muscle environment.

Ch 3: Tissue engineering a 3D muscle model: Mechanical stimulus

Introduction

To date there have been a number of attempts to tissue engineer skeletal muscle *in vitro* and many researchers have used a "dynamic culture approach" in bioreactors (summarised in Table 5), based on the hypothesis that contractile functional skeletal muscle tissue will fully develop *in vitro* only if subjected to mechanical strains that prevail during normal *in vivo* organogenesis and growth (Riboldi *et al.*, 2005).

Mechanical forces play an important role in modulating the growth of a number of different tissues including skeletal muscle, smooth muscle, cardiac muscle, bone, endothelium and lung (Vandenburgh, 1992). Both passive and active mechanical forces play a role in the transition of skeletal muscle from the embryonic to the mature state (Goldspink *et al*, 1992). Further, directed mechanical tension is important to organise myoblasts into functional aligned myotubes and provides a stimulus for the expression of mature isoforms of myofibrillar proteins (Tatsumi *et al*, 2001).

It has been shown that mechanical stimulation of tissue-cultured skeletal muscle cells causes changes similar to those seen when *in vivo* skeletal muscle is mechanically loaded (Powell *et al.*, 2002). Vandenburgh *et al.* (1991) showed that repetitive stretch/relaxation of avian skeletal muscle cells *in vitro* by patterns that simulate *in vivo* skeletal growth and exercise altered the structural organization of the constructs.

Unidirectional stretch followed by repetitive stretch of avian bioartificial muscles was shown to lengthen, orient, and organize the myofibers compared with static cultures (Vandenburgh *et al.*, 1991). Similarly, stress loading was shown to improve the orientation and density of rodent myofibers cultured in collagen gels (Okano and Matsuda, 1997). Cheema *et al.* (2004) showed that when C2C12 myoblasts were grown in 3D culture the insulin-like growth factor (IGF-I; which initiates the fusion of myoblasts to form myotubes (Florini *et al.*, 1991)) gene was upregulated by a single ramp load (10% strain of 1-hr duration). Mechanical stimulation increases cellular proliferation (Vandenburgh *et al.*, 1989), myotube organization (Collinsworth *et al.*, 2000, Vandenburgh, 1982; Vandenburgh *et al.* 1991; Okano and Matsuda, 1997), and extracellular matrix (ECM) composition (Vandenburgh *et al.* 1991).

Powell *et al.* (2002) were the first group to attempt to tissue engineer skeletal muscle using primary human cells derived from the vastus lateralis. The primary human muscle cells were expanded in tissue culture, and statically tissue engineered (in a collagen-matrigel^{TM} construct) into human bioartificial muscles (HBAM). The HBAMs were maintained in growth medium for 3 days, then the medium was changed to low serum fusion medium for 5 days. Cheema *et al.* (2003) also statically tissue engineered C2C12 bioartificial muscles (in a collagen constructs) over a 7-day period and monitored mRNA levels, reporting a transient elevation of IGF gene expression on day 3.

Literature	Cell Source	Scaffold material	Loading conditions
Vandenburgh *et al.*, 1988	Avian Skeletal muscle	Collagen coated silastic membrane	Three stretch regimens: 1. Slow continuous ramp stretch of the silastic substrator at 0.35mm/hour for 24 hours 2. Followed by a slower 0.09mm/hour for an additional 24 hours. 3. Then a cyclical stretch-relaxation of the substratum at 0.5 Hz for 10s followed by a 150s rest period for 40-48 hrs. Total substratum stretch was 3.5% or 17% by using prongs at different heights.
Vandenburgh *et al.*, 1991	Avian Skeletal muscle	Collagen coated silastic membrane	Five 20% strain substratum stretches and relaxations during a 20s period followed by 10 s rest. Repeated 3 times followed by a variable rest period of 0-30 minutes.
Okano and Matsuda, 1998	C2C12	Type I collagen gel	Cyclical stretching (frequency 60rpm; amplitude 5%) for up to 4 days.
Collinsworth *et al.*, 2000	C2C12	Matrigel coated silastic membrane	Average strain rate 2.36%/day for 4 days or 1.77%/day for 6 days resulting in 9.5 or 10.6% respectively.
Powell *et al.*, 2002	Human skeletal muscle	Matrigel:collagen (1:6) gel	500μm/day for 4 days (~3.5 μm every 10 minutes) Total unidirectional stretch over 4 days of 10% initial length.
Cheema *et al.*, 2004	C2C12	Type I collagen gel	Three ramp regimens; 10% length change over 12 hrs, 1 hr and 10 minutes. Three cyclical loading regimens; 1, 5 and 10 cycles/hr at strain rates of 4, 20 and 40% / hr respectively.
Auluck *et al.*, 2005	Human skeletal muscle	Collagen sponges	Rapid ramp stretch or cyclical ramp strain with 7.5% or 15%

Table 5: Summary of selected studies illustrating the range of *in vitro* dynamic loading regimens used to develop tissue engineered skeletal muscle constructs.

Powell *et al.* (2002) demonstrated that mechanical stimulation improved tissue engineered HBAMs; mechanical stretching began at 24 hrs and the HBAMs were stretched 500 μm/day for 4 days; at step intervals of ~3.5μm every 10 min (with a total unidirectional stretch over 4 days of 10% of the initial length), resulting in significant increases in both myofiber diameter and myofiber density (Powell *et al.*, 2002).

In this study the tensioning-Culture Force Monitor (t-CFM) was used to apply mechanical loading regimens (Eastwood *et al.*, 1998; Brown *et al.*, 1998; Prajapati *et al.*, 2000; Mudera *et al.*, 2000; Cheema *et al.*, 2004) to the 3D MDC cultures in a two-fold attempt to develop a tissue engineered skeletal muscle model and to monitor how mechanical cues effects the gene expression of markers of differentiation (myogenin), phenotype (slow MyHC-β and fast MyHC-IIX/D) and matrix remodelling (MMP-2). The objective of this study was to statically engineer the heterogenous MDC 3D culture (presented in chapter 2) over 3 days and then apply two continuous ramp loading regimens (1. Slow (10% strain applied over a 1 hour period); 2. Fast (10% strain applied over a 10 minute period)). The loading regimens implemented in this study were consistent with previously reported optimal loading conditions comparable to physiological conditions (Cheema et al., 2004; Auluck et al., 2005).

The manner in which cells respond to different mechanical environments has attracted wide interest, both the level of the applied load and the rate of loading are known to be important in eliciting a specific cellular response (Bader and Lee, 2005; Karamichos, 2006). Muscle is a tissue in which gene expression is regulated to a large extent by mechanical signals (Goldspink *et al.*, 1992). However, little is known

about the molecular mechanisms by which muscle tension regulates transcription of specific genes and future studies will depend greatly on the use of tissue culture mechanical stimulator model systems (Vandenburgh, 1992). Based on the literature presented that mechanical stimulation improves tissue engineered muscle constructs, *the first aim of this study was to monitor molecular output using RT-PCR to quantitate the maturation and development of the muscle construct. To test the hypothesis that mechanical stimulation would elicit an up-regulation of the muscle regulatory factor myogenin, indicative of increased myogenic cell differentiation.*

An important characteristic of skeletal muscle is its plasticity. Muscle fibers are characterized by an inherent adaptability to environmental alterations by changing from one fiber type into another (Adams *et al.*, 1993; Goldspink, 1998; Oishi *et al.*, 1998). They are versatile elements, which are capable of modifying their structure and properties to physiological and functional demands (Pette & Staron, 1990). This is reflected in the composition and level of expression of myosin heavy chain (MyHC) isoforms within the muscle; which represent the best markers reported of muscle fiber adaptive changes (Schiaffino & Reggiani, 1996; McKoy *et al.*, 1998; Agbulut *et al.*, 2003).

Skeletal muscle plasticity has been studied extensively by myosin isoform transitions (d'Albis *et al.*, 1989, 1990; Bacou *et al.*, 1996; McKoy *et al.*, 1998; Baldwin and Haddad, 2001). Various factors such as development, innervation, increased and decreased neuromuscular activity, physical activity (mechanical loading and unloading), hormones and aging have been shown to influence the phenotypic expression of skeletal muscle (Sullivan *et al.*, 1995; Schaiffino and Reggiani, 1996;

Pette and Staron, 2000; Baldwin and Haddad, 2001; Agbulut *et al.*, 2003). These factors can induce MyHC isoform expression heading in the direction of either fast-to-slow or slow-to-fast. Increased neuromuscular activity, mechanical loading and hypothyroidism are conditions that induce fast-to-slow transitions, whereas reduced neuromuscular activity, mechanical unloading and hyperthyroidism cause transitions in the slow-to-fast direction (Pette and Staron, 2000).

Periasamy *et al.* (1989) reported that the change in the pattern of myosin isozymes protein expression during skeletal-muscle adaptation to work overload was a consequence of changes in specific myosin heavy chain (MyHC) mRNA levels. Physical activity can alter muscle phenotype and contractile function by switching on one gene subset and repressing another (Goldspink, 1998). The dynamic nature of skeletal muscle in response to altered functional demands requires switches in gene expression of rapidity and magnitude in response to a changing cellular environment (McKoy *et al.*, 1998).

In summary, skeletal muscle changes in phenotype result from differential gene expression in response to mechanical signals (Goldspink *et al.*, 1992). A study by McKoy *et al.* (1998) described the switching of all the major MyHCs at the mRNA level as an animal matures and individual muscles become adapted to perform highly specialized and diverse functions. They also demonstrated that changes in MyHC gene expression at the mRNA level correlated strongly with those observed at the protein level (McKoy *et al.*, 1998). This indicates that the determination of muscle phenotype is controlled at the level of gene transcription (Goldspink, 1998; Baldwin and Haddad, 2001). The objective of this part of the study was to use the statically

developed 3D muscle construct to investigate skeletal muscle plasticity in response to specific mechanical loading regimens. *The aim was to monitor the effect of the mechanical loading regimens on the MyHC gene expression (slow MyHC-β and fast MyHC-IIX/D). To test the hypothesis that mechanical cues will cause a switching of MyHC gene expression with a fast-to-slow transition, i.e. a relative up-regulation of slow MyHC- β mRNA and a down-regulation of fast MyHC-IIX/D mRNA.*

The way in which cells respond to mechanical signals is a new area of physiology and the evidence indicates that this involves the cytoskeleton and the ECM (Goldspink, 1998). In skeletal muscle, an important interplay between muscle cells and the ECM is present, and it has been suggested that *in vivo* adult human muscle there are common mechanical signaling pathways to stimulate contractile and ECM components (Kjær, 2004). Currently, little is known about how the muscle ECM interacts with proliferating and differentiating muscle cells to influence the formation and stabilization of skeletal muscle (Velleman *et al.*, 1998). Further, it is not known how the surrounding soft tissues are activated and what the cellular processes are, that convert the mechanical signals into those that will influence tissue remodeling and adaptation (Auluck *et al.*, 2005).

Signals from mechanical loading will initiate a cascade leading from gene expression, transcription, translation, and posttranslational process modification to the integration of events to provide protein synthesis in the ECM (Kjær, 2004). Changing demands in skeletal muscle (e.g. increased or decreased contractile activity) promotes remodeling of the ECM (Carmeli *et al.*, 2005) and mechanical stress is a potent stimulus for ECM reorganization (Ruwhof *et al.*, 2000). The ECM turnover is

influenced by physical activity and it has been shown both collagen synthesis as well as activity of degrading MMP enzymes increase with mechanical loading (Prajapati *et al.*, 2000; Mudera *et al.*, 2000; Kjær, 2004; Auluck *et al.*, 2005; Karamichos, 2006). *The final aim of this study was to investigate the effect of mechanical loading regimens on skeletal muscle ECM remodeling by monitoring the in vitro expression of the mechanoresponsive gene MMP-2 in tissue engineered human muscle constructs. To test the hypothesis that MMP-2 expression, and hence ECM remodeling, is up-regulated in response to mechanical loading and is dependent upon the rate of loading to which the muscle construct is subjected.*

Materials and Methods

As previously described the CFM (Eastwood et al., 1994) fundamentally consists of a force transducer at one end and an anchoring point at the other, providing the axis for measurement of the uni-axial endogenous force generated as the embedded cells contract the tethered, free floating (practically frictionless) collagen construct. The CFM was further developed by Eastwood *et al.* (1998) into a computer driven tensional loading device to apply exogenous mechanical loading regimens.

The t-CFM utilizes the same force transducer, data collection system, and culture well as the CFM. However, the additional feature of the t-CFM is the stepper motor, which can be programmed via a computer to apply varying mechanical loads to the collagen gel by moving the base uniaxially (Figure 37). Initial positioning of the culture well during the experimental setup is through the moveable "X-Y" table mounted onto the motorized base (Eastwood *et al.*, 1998).

The t-CFM bioreactor enabled precise uni-axial mechanical loads to be applied to the MDC seeded collagen constructs, whilst simultaneously recording the total mechanical load (Figure 40; Ramp loading regimens). The analogue output signal was amplified, digitised and process using LabView software (National Instruments, v.6, Newbury, UK) and allowed accurate definition of the magnitude and type of mechanical loading to which the matrix was subjected (Eastwood *et al.*, 1998).

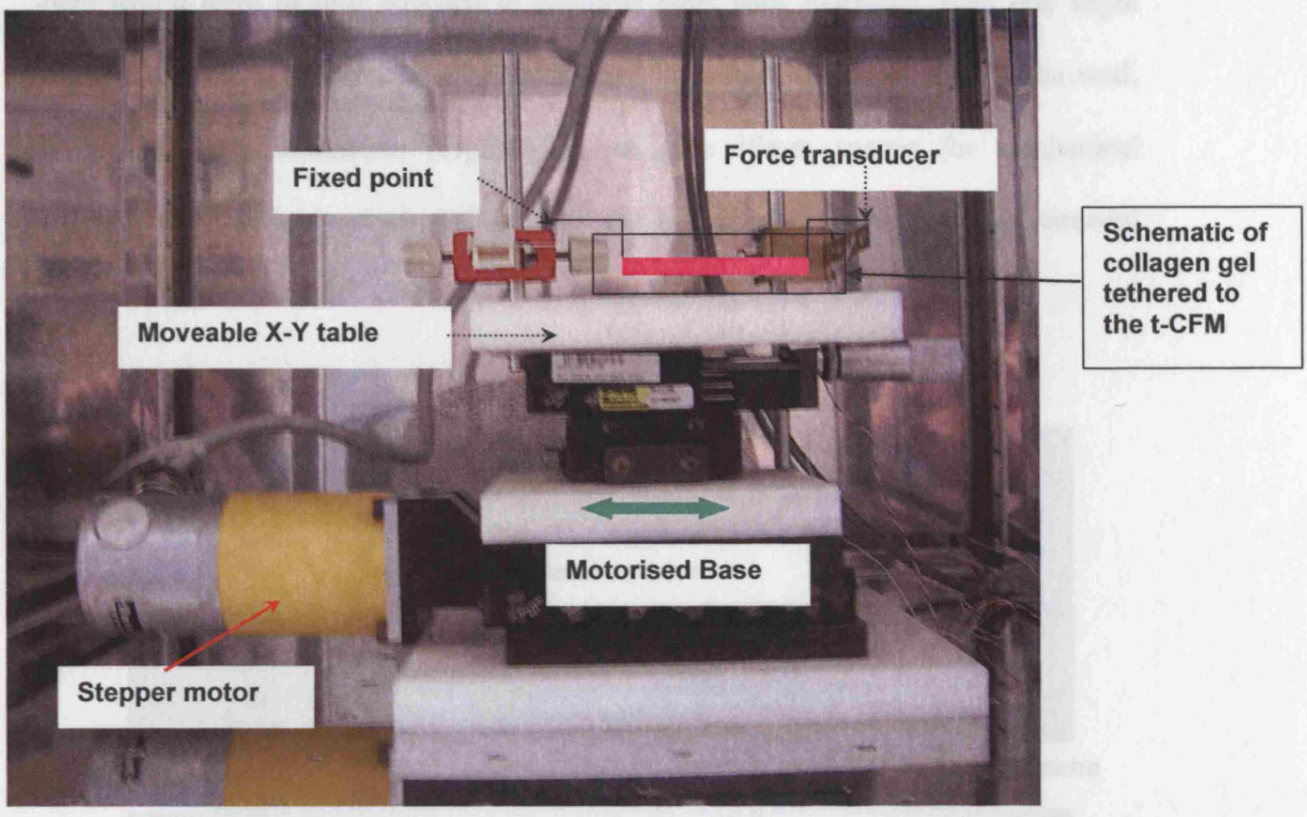


Figure 37: This picture shows the t-CFM set up. The additional feature of this set up is the stepper motor (indicated by red arrow) which is attached to the base. This motor can be programmed via a computer, to apply varying mechanical loads to the collagen gel by moving the base uni-axially (indicated by the green double headed arrow).

Collagen gels were cast in the same way as for the CFM set up between 2 floatation bars which were in turn attached to stainless steel wire A-frames, with one slight difference, plastic spacer bars were positioned at each of the short ends of the well, before collagen incubation (Figure 38), to give 10mm spaces for mechanical extension (Karamichos *et al.*, 2006). Once the gel had set the spacers were removed from the well.

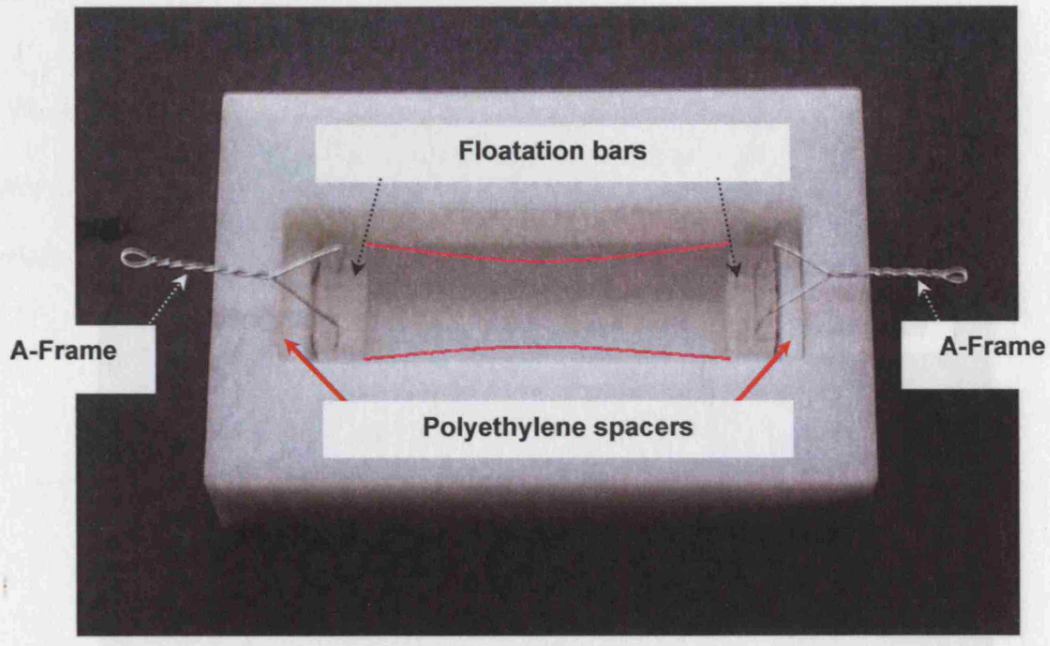


Figure 38: Before collagen incubation (indicated by pink lines), two plastic spacer bars were positioned at each of the short ends of the well (indicated by red arrows), to give 10mm spaces for mechanical extension. Spacers were removed when added gel was set.

The collagen gels defined in chapter 2 (i.e. MDCs seeded at a density of 5 million/5ml collagen gel) were developed by statically engineering (Powell *et al.*, 2002; Cheema *et al.*, 2003) the constructs between two fixed points in a sterile and closed

environment (Figure 39) for 3 days. To induce myogenic cell differentiation, the standard growth medium was changed to differentiation medium after 24 hours. No strain was applied to the static control cultures, maintained under endogenous tension with no external loading. The constructs which were to be subject to mechanical loading were tethered to the t-CFM at 48 hours and exogenously loaded at 60 hours. The loading regimens were designed to apply ramp loading to the cultures using the X-ware software X150 (Parker Automation, California, USA).

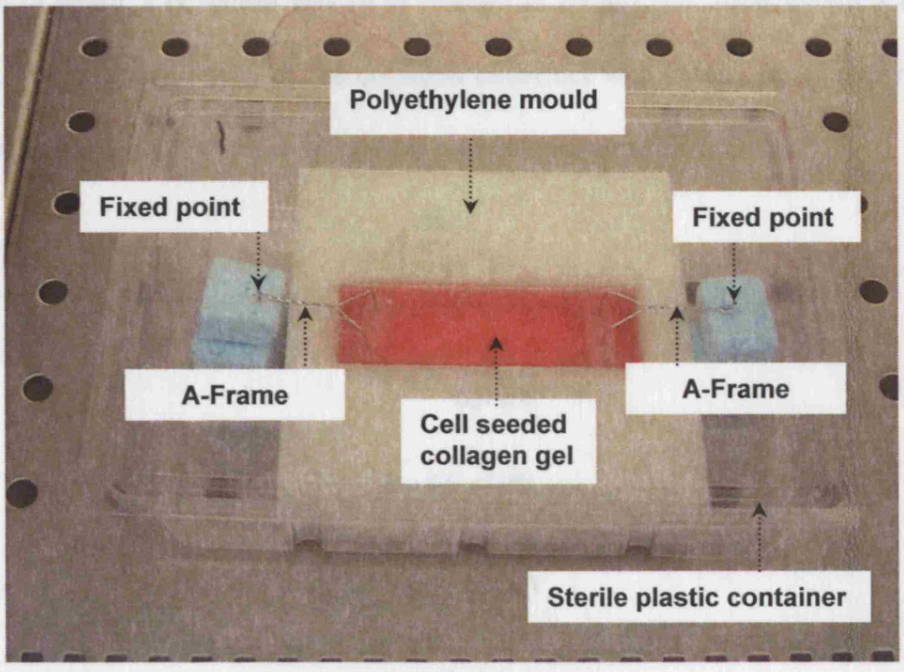


Figure 39: The primary human MDC 3D cultures were statically developed in static culture for 3 days to promote differentiation of myoblasts to form myotubes.

Each revolution of the motor related to a certain distance by which the stage moved. The motor had a step angle of 1.8° and a default set up of 4000 steps (equivalent to one revolution). Further using a screw pitch of 0.5mm/revolution we could calculate the distance the stage needed to move to apply 10% strain to the constructs.

Strain is classically defined as:

$$\varepsilon = \frac{\Delta l}{l}$$

Where ε is strain, l is length and Δl is change in length (i.e. final length – original length). The original length of the collagen construct was 65mm, so to apply a 10% strain distance the stage was to be moved was calculated to be 6.5mm.

$$\varepsilon = \frac{\Delta l}{l}$$

$$10\% = \frac{\Delta l}{65mm}$$

$$\Delta l = 65 \times 10\%$$

$$\therefore \Delta l = 6.5mm$$

Given,

$$1rev = 0.5mm$$

$$\therefore \Delta l = 6.5mm = 13rev$$

Further,

$$1rev = 4000steps$$

$$\therefore \Delta l = 13rev = 52000steps$$

Therefore, to apply 10% strain the motor was programmed to move 52000 steps. Two ramp loading regimens were applied (Figure 40); (a) Fast stretch (over 10 minutes) and (b) slow stretch (over 1 hour). To programme the motor the rate of strain (Velocity) had to be determined as revolutions/second. For the two loading regimens velocity (V) was calculated as distance (d) over time (t) as follows:

(a) Fast

$$V = \frac{d}{t}$$

$$V = \frac{6.5mm}{10\,\text{min}}$$

$$V = \frac{13revolutions}{600\,\text{sec}onds}$$

$$\therefore V = 0.02167revs/s$$

(b) Slow

$$V = \frac{d}{t}$$

$$V = \frac{6.5mm}{1hr}$$

$$V = \frac{13revolutions}{3600\,\text{sec}onds}$$

$$\therefore V = 0.003611revs/s$$

After these components of the loading regimen had been determined, the t-CFM motor was programmed. An example program to apply slow stretch, i.e. 10% ramp load over 1 hour is given:

```
1ON             ; Energise drive
T43200          ; Time delay (wait 12 hours)
A1              ; Acceleration rate constant
V0.003611       ; Velocity (revolutions/second)
MN              ; Sets the mode to normal preset distance
D-52000         ; Distance measured in steps (start loading 10% strain)
G               ; Go
N               ; End
```

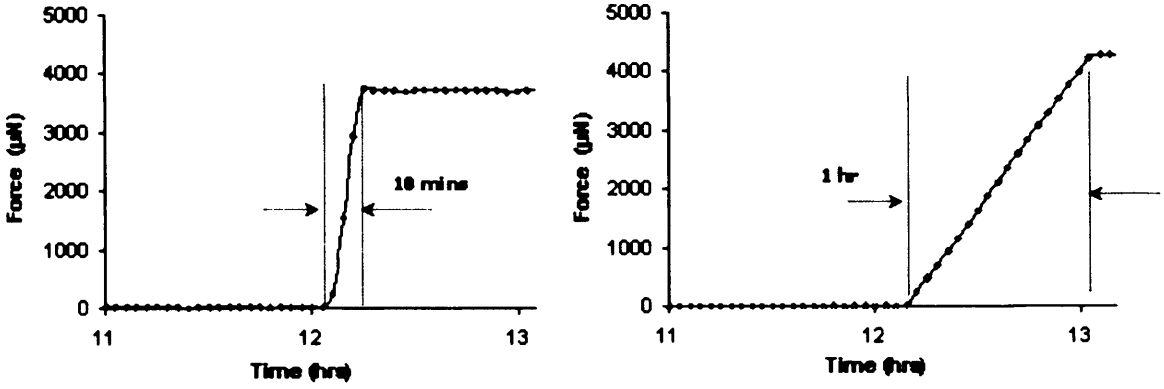


Figure 40: The patterns of ramp loading regimens applied to MDC seeded 3D collagen constructs cultured for 3-days. (a) Fast ramp regimen: 10% strain (6.5mm displacement) was applied to the cultures over a 10 minute period. (b) Slow ramp regimen: 10% strain (6.5mm displacement) was applied to the cultures over a 1 hour period.

After application of ramp load over a 1-hour or 10-minute period, cultures remained held at 10% strain over 11 hour or 11 hour and 50 minutes (respectively) after load was applied. Gels were then untethered from the t-CFM at 72 hours and processed for RNA extraction and RT-PCR. Using the $2^{-\Delta\Delta Ct}$ method, the results are presented as the fold change in gene expression normalized to an endogenous reference and relative to a control. The relative control in this study was a heterogenous MDC seeded (5million/5ml gel) collagen construct after 24 hours tethered to the CFM (Chapter 2).

Results

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a catalytic enzyme involved in glycolysis and is expressed in all cells. It is important that the internal control gene is properly validated for each experiment to determine that gene expression is unaffected by the experimental treatment (Livak and Schmittgen, 2001). Figure 41 shows the expression of GAPDH under each loading regimen. No significant difference (p=0.1354) was found in GAPDH expression between static and loading conditions. Therefore, GAPDH was used as the reference (housekeeping) gene in this study against which all other gene expression markers were compared.

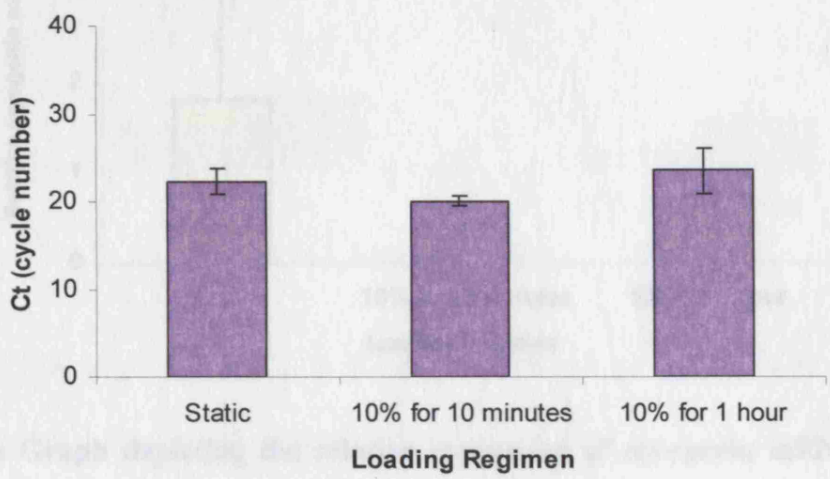


Figure 41: The effect of the different mechanical loading regimens on GAPDH mRNA expression levels in 3D MDC seeded collagen constructs after 3 days (72 hrs) in culture. There was no significant difference (p=0.1354) in gene expression so GAPDH was used as the housekeeping gene in this study. Standard deviation used for error bars (n=3).

To test the hypothesis that mechanical loading would improve the statically engineered tissue constructs and elicit a corresponding gene response that would enhance myogenic differentiation, myogenin mRNA was quantified. The results showed that mechanical stimulation did not cause an up-regulation of myogenin (Figure 42). Both the fast and slow ramp loading rates, at 10% strain, to which the construct was subject, caused a down regulation (1.5-fold and 0.9 fold change respectively) of myogenin relative to the static control. These changes in gene expression were not significantly different (p=0.1767) and the mechanical loading regimes applied did not promote myogenic cell differentiation.

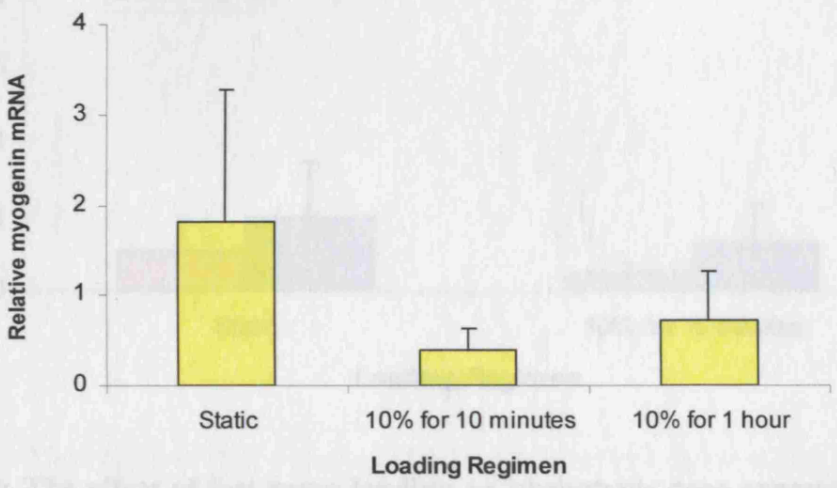


Figure 42: Graph depicting the relative expression of myogenin mRNA before (Static) and after application of ramp loading (Fast; 10% for 10 minutes and Slow; 10% for 1 hour) regimens. Relative mRNA expression levels were determined by RT-PCR. Error bars represent standard deviation (Static (n=3) sd ± 1.46; 10% for 10 minutes (n=3) sd ± 0.23; 10% for 1 hour (n=3) sd ± 0.55). By ANOVA statistical analysis these results were not significantly different (p=0.1767).

To investigate whether fast ramp loading caused a switch in phenotype, gene expression of the relative MYHC isoforms were monitored. The fast ramp loading regime had no effect on either the fast MyHC-IIX/D or the slow MyHC-β mRNA expression. Further, the results indicated no significant difference (p=0.6047) in the MyHC gene expression between the static and fast ramp loaded cultures (Figure 43). Therefore, this loading regimen did not cause a change in MyHC gene expression.

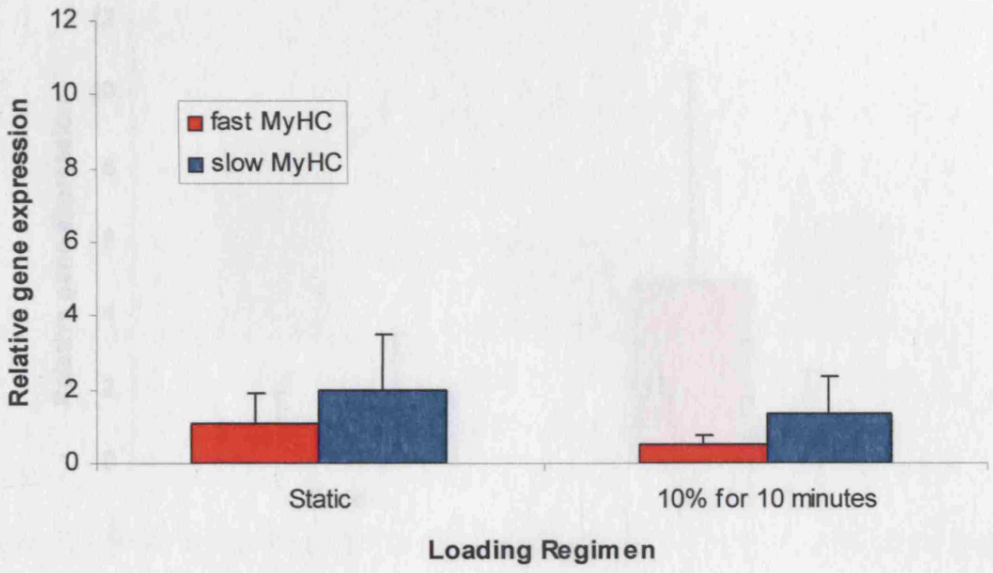


Figure 43: The effect of fast ramp loading on phenotypic gene expression in 3D MDC cultures. This graph depicts the relative expression of the fast MyHC-IIX/D (red) and the slow MyHC-β (green) mRNA (using the static culture as the control) after application of 10% strain for 10 minutes. Error bars represent the standard deviation (Fast MyHC-IIX/D; Static (n=3) sd ± 0.80, 10% for 10 minutes (n=3) sd ± 0.20 and Slow MyHC-β; Static (n=3) sd ± 1.57, 10% for 10 minutes (n=3) sd ± 1.04). By ANOVA statistical analysis these results were not significantly different (Fast MyHC-IIX/D, p=0.3028; Slow MyHC-β, p=0.6047).

However, the slow ramp loading regime elicited a 4-fold (sd ± 5.63) up-regulation of the MyHC fast isogene, and a 1-fold (sd ± 0.69) down-regulation of the slow MyHC isogene (Figure 44). These results suggest that different rates of strain alter MYHC mRNA expression and this 3D muscle construct could be used as a model to simulate skeletal muscle plasticity.

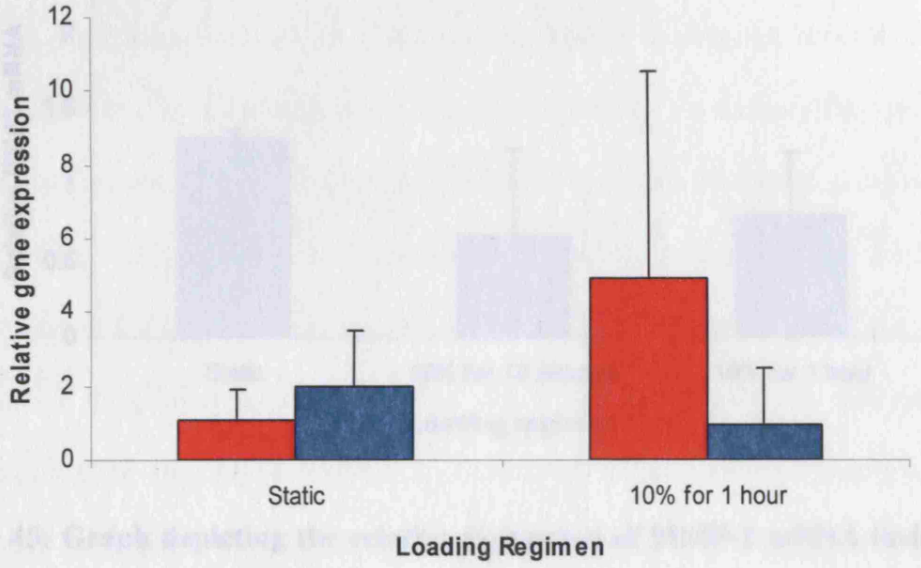


Figure 44: The effect of slow ramp loading on phenotypic gene expression in 3D MDC cultures. This graph depicts the relative expression of the fast MyHC-IIX/D and the slow MyHC-β mRNA (using the static culture as the control) after application of 10% strain for 1 hour. Error bars represent standard deviation (Fast MyHC- IIX/D; Static (n=3) sd ± 0.80; 10% for 1 hour (n=3) sd ± 5.63 and Slow MyHC-β; Static (n=3) sd ± 1.57; 10% for 1 hour (n=3) sd ± 0.69). By ANOVA statistical analysis these results were not significant (Fast MyHC, p=0.3096; Slow MyHC, p=0.4716).

The final aim of this study was to monitor the response of MMP-2 gene expression to strain as a marker of ECM remodelling. Under the mechanical loading regimens imposed on the muscle construct the results show no significant (p=0.1184) change in MMP-2 mRNA expression. Further, the rate of strain did not significantly effect MMP-2 mRNA expression.

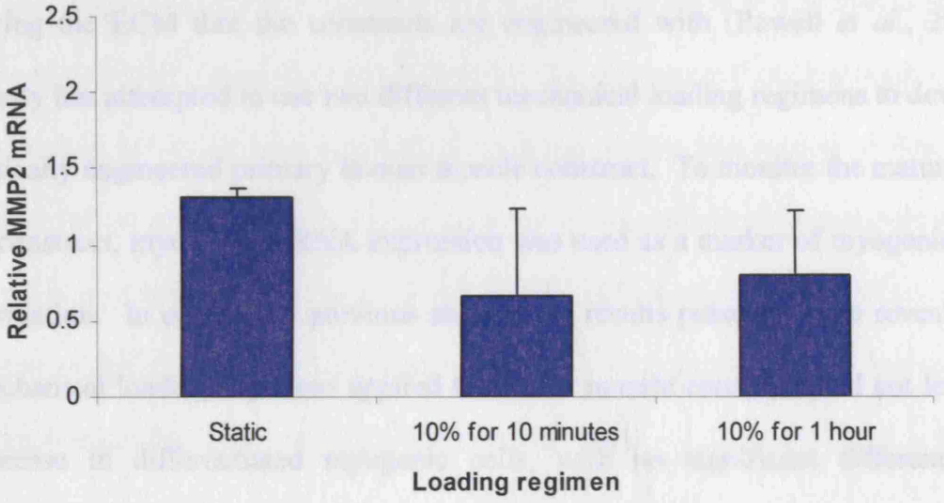


Figure 45: Graph depicting the relative expression of MMP-2 mRNA (using the static culture as the control) after application of ramp loading (Fast 10% for 10 minutes and Slow 10% for 1 hour) regimens. Relative mRNA expression levels were determined by RT-PCR. Values representing the amounts of MMP2 mRNA were normalised to GAPDH. Error bars represent standard deviation (Static (n=3) sd ± 0.06; 10% for 10 minutes (n=3) sd ± 0.57; 10% for 1 hour (n=3) sd ± 0.42). By ANOVA statistical analysis these results were not significant (p=0.1184).

These results suggest that the current loading regimens do not elicit the gene response necessary to induce myogenic differentiation or matrix remodelling. Mechanical cues did not lead to further maturation of this 3D muscle construct.

Discussion

Mechanical loading improves tissue engineered skeletal muscle constructs (Vandenburgh *et al.*, 1988, 1991; Okano and Matsuda, 1998; Collinsworth *et al.*, 2000; Powell *et al.*, 2002; Cheema *et al.*, 2004). However, to date tissue engineered skeletal muscle is still far removed from *in vivo* skeletal muscle and further improvements may be possible by changing the mechanical stimulation pattern or improving the ECM that the constructs are engineered with (Powell *et al.*, 2002). This study has attempted to use two different mechanical loading regimens to develop the statically engineered primary human muscle construct. To monitor the maturation of the construct, myogenin mRNA expression was used as a marker of myogenic cell differentiation. In contrast to previous studies, the results presented here reveal that the mechanical loading regimens applied to the 3D muscle constructs did not lead to an increase in differentiated myogenic cells, with no significant difference in myogenin mRNA expression between the static and loaded muscle constructs. This study has established that under the defined conditions of this muscle model, mechanical loading did not improve the tissue engineered construct.

Skeletal muscle is a tissue that possesses an intrinsic ability to phenotypically adapt to the type of physical activity that it is required to perform, i.e. remodelling by physical activity (Yang *et al.*, 1997; Carmeli *et al.*, 2005). The ability of adult muscle cells to modulate the properties of contractile apparatus in response to environmental and physiological stimuli even after terminal differentiation illustrates their remarkable plasticity (Periasamy *et al.*, 1989). Pette and Staron (2000) reported that fiber type transformations represent highly coordinated processes of gene up-regulation and down-regulation, concluding that future research will be needed to elucidate the

molecular elements and mechanisms underlying fibre type determination and transformation (Pette and Staron, 2000). When subjected to stretch, skeletal muscle apparently adapts to a more postural type of role by expressing the slow myosin isoform and repressing the fast myosin genes (Goldspink *et al.*, 1992). This part of the study investigated the effect of external mechanical loads on the *in vitro* 3D muscle constructs to simulate skeletal muscle plasticity. The results show that gene expression was dependent on the type of strain applied as the slow mechanical stretch did not effect MyHC gene expression, but the fast mechanical loading regimen did elicit a change in MyHC gene expression. Suggesting that mechanical loading can cause a switch of MyHC gene expression, however, the slow-to-fast transition with a relative up-regulation of the fast MyHC-IIX/D mRNA, monitored here was not consistent with the *in vivo* finding that mechanical loading induces a fast-to-slow transition (Goldspink *et al.*, 1992; Pette and Staron, 2000).

The need for physiologically relevant models with which to study muscle cell biology has led to studies in which specific mechanical forces and ECM materials are combined to recreate a complex environment. These types of studies are important in the field of tissue engineering and regenerative medicine, where the ability to understand and reconstitute the physiological environment is key to developing biologically based tissue substitutes (Stegemann *et al.*, 2005). The mechanisms by which cells detect and respond to the mechanical environment are termed mechanotransduction pathways (Bader and Lee, 2005). Very little is known about the mechanotransduction mechanism involved and what muscle regulatory factors may be involved that induce changes in transcription of specific genes (Yang *et al.*, 1997). A better understanding of the relationship between initial application of load and

effector cell response is necessary for the appreciation of the growth and adaptation of normal and pathological tissues and will be of use in the development of tissue engineered repair systems (Bader and Lee, 2005). As previously stated, MMP-2 is a known mechanoresponsive gene (Bolcato-Bellemin *et al.*, 2000; Mudera *et al*, 2000; Auluck *et al.*, 2005) that has been used as a marker of ECM remodeling and was monitored in this study to investigate the cellular response to mechanical loading, specifically, does exogenous mechanical loading elicit a gene response that stimulates matrix remodeling.

Cheema (2004) noted that when muscle cells (C2C12s) were grown in 3D culture and subjected to mechanical strains comparable to those generated during early myogenesis, total IGF-I mRNA expression was optimally stimulated in the region of 10% strain/hour for both ramp and cyclical loading regimens, though the magnitude of stimulation was much greater for ramp loads (Cheema *et al.*, 2004). Concluding that, not only does ramp loading invoke a different response, but the strain rate is also critical in determining the type and magnitude of mRNA expression (Cheema *et al.*, 2004). Similarly, Auluck *et al.* (2005) noted that MMP-2 expression was found to be significantly higher in those skeletal MDC cultures strained continuously, compared to cyclical strain, and in those strained at 15% compared to 7.5%, concluding that skeletal muscle is more likely to respond to a change in the environment if it is carried out with a single activation as opposed to a step-by-step activation. Further, Asunama *et al.* (2003) demonstrated that the in vitro response of vascular smooth muscle cells is dependent on the strain regimen, where continuous mechanical strain led to enhanced matrix degradation via an increased expression of MMP-2, however, cyclic strain decreased the expression of MMP-2 mRNA. The type of mechanical load

applied to cultures is important in determining changes in gene expression and in this study, although similar continuous ramp loading conditions were applied MMP-2 mRNA expression was not altered by the mechanical strain.

Carmeli *et al.* (2005) explored the effect of exercise on MMP-2 activity and reported that low intensity exercise did not alter the expression of MMP-2 in any of the muscles investigated (Carmeli *et al.*, 2005). Yasuda *et al.* (1998) reported that mesangial cells induced by stretch/relaxation showed biphasic changes in MMP-2 activity; in the first 24 hours after stretch MMP-2 mRNA levels were significantly decreased, however, after more prolonged periods of stretch/relaxation, increases in MMP-2 mRNA levels were noted. Together with the results presented in this study, these observations emphasize the complexity of the interplay between mechanical signals and cellular response.

It is important to note that much of the data reported in the literature provides phenomenological observation, specific to a particular model system, which may not be easily translated to other model systems. Only by conducting systematic studies, involving the alteration of one variable at a time, can underlying mechanistic parameters be defined leading to the derivation of predictive strategies suitable for the optimisation of bioreactor systems (Bader and Lee, 2005). Future research should be carried out on this muscle construct to identify the specific mechanical stimuli required to elicit a cellular response that could be harnessed to develop sophisticated tissue engineered muscle constructs.

Conclusions

- The mechanical loading regimens implemented in this study did not elicit an up-regulation of the muscle regulatory factor *myogenin* and did not induce myogenic cell differentiation within the tissue engineered 3D muscle constructs.

- MyHC gene expression was not effected by the fast ramp loading regimen. However, the slow ramp loading condition caused a switch in gene expression in the slow-to-fast direction, with a relative up-regulation of fast MyHC-IIX/D.

- MMP-2, the marker of ECM remodeling was not up-regulated in response to the mechanical loading regimens applied. MMP-2 mRNA expression was unaffected by the exogenous loading conditions.

Ch 4: Tissue engineering 3D muscle model: The effect of high cell seeding density

Introduction

Skeletal muscle tissue engineering represents a scientific approach that attempts to create and regenerate skeletal muscle tissue. As previously stated, skeletal muscle consists of long bundles of densely distributed and highly oriented cells called muscle fibers, each of which is a highly differentiated multinucleated cell derived from myoblasts. The muscle fibers in native skeletal muscle are packed closely together in a thin layer of extracellular matrix composed mainly of collagen to form a well-organized tissue with high cell density and orientation. Tissue criteria for engineering muscle constructs structurally bio-mimicking native muscle (in terms of their ability to mediate generate force) must include high cell density, a high degree of cell fusion (or extensive myotube formation) and a high degree of unidirectionality (Okano and Matsuda, 1998).

Mao *et al.*, (2006) reported that cell density matters in the tissue engineering of the mandibular condyle and stated that a dissatisfactory feature in their previous work was suboptimal tissue maturation. (Alhadlaq and Mao, 2003; Alhadlaq *et al.*, 2004) They then attempted to improve tissue maturation and osteochondral integration by increasing the cell encapsulation density from the previous 5×10^6cells/mL to 20×10^6cells/mL; at this high density the tissue-engineered mandibular joint condyle showed evidence of maturation with phenotypic growth of both cartilage and bone-like tissues (Alhadlaq and Mao, 2005).

A 'simplified method' for tissue engineering skeletal mammalian organoids *in vitro* by seeding myoblasts at a high plating cell density was published by Shansky *et al.* (1997). Primary myoblasts from the forelimbs and hindlimbs of rat neonates were suspended in a 1:6 solution of Matrigel:collagen (Type 1) and plated into tissue culture wells at a concentration of 4 million/0.750mls (5.33 million/ml) gel (Shansky *et al.*, 1997). Bach *et al* (2003) also had a working principle for the preparation of functional muscular tissue based on myoblasts being cultured at high cell density (150,000 cells in 125µl growth medium; 1.2 million cells/ml) and seeded in 125µl of fibrinogen.

Further, non-woven arrays of polyglycolic acid (PGA) fiber mesh sheets (1cm × 1cm with fiber diameter approximately 12 μm) have been used as a scaffold to tissue engineer skeletal muscle, in which a high cell seeding density was paramount, constructs were seeded with myoblasts at a density of 2.75–4.75 million cells/polymer (Saxena *et al.*, 2001). Thorrez *et al.* (2008) similarly seeded cells on 13mm poly-lactide-co-glycolide PLG scaffolds at high densities ranging from 4-16 million in a recent attempt to tissue engineer skeletal muscle.

The objective of this study was to tissue engineer skeletal muscle constructs using:

1) High cell density to increase the probability of cell fusion leading to multinucleated myotube formation; and

2) Predictive unidirectional cellular alignment facilitating myogenic cell fusion and generation of force.

In vivo, in developing muscle the cues directing the orientation of myotube formation are not well understood, however in repair of muscle, it is evident that the existing myofibres may provide cues along which the myoblasts align and fuse (Clark *et al.*, 1997; Cheema, 2004; Shah *et al.*, 2005). It is possible to align myotube formation through providing cues such as physical grooves in culture dishes, and culturing of myoblasts in collagen gels and fibronetin gels, where the generation of isometric tension in a uni-axial plane provides a direction for orientation (Iseval *et al.*, 1980; John *et al.*, 1980; Turner *et al.*, 1983; Cheema, 2004). The CFM system provides unidirectional cellular orientation; the cells align themselves along the axis of principle strain (Figure 46; adapted from Eastwood *et al.*, 1998), generated by the endogenous tension developed as cells contract the matrix (Eastwood *et al.*, 1998; Mudera *et al.*, 2000; Cheema *et al.*, 2003; Karamichos *et al.*, 2007).

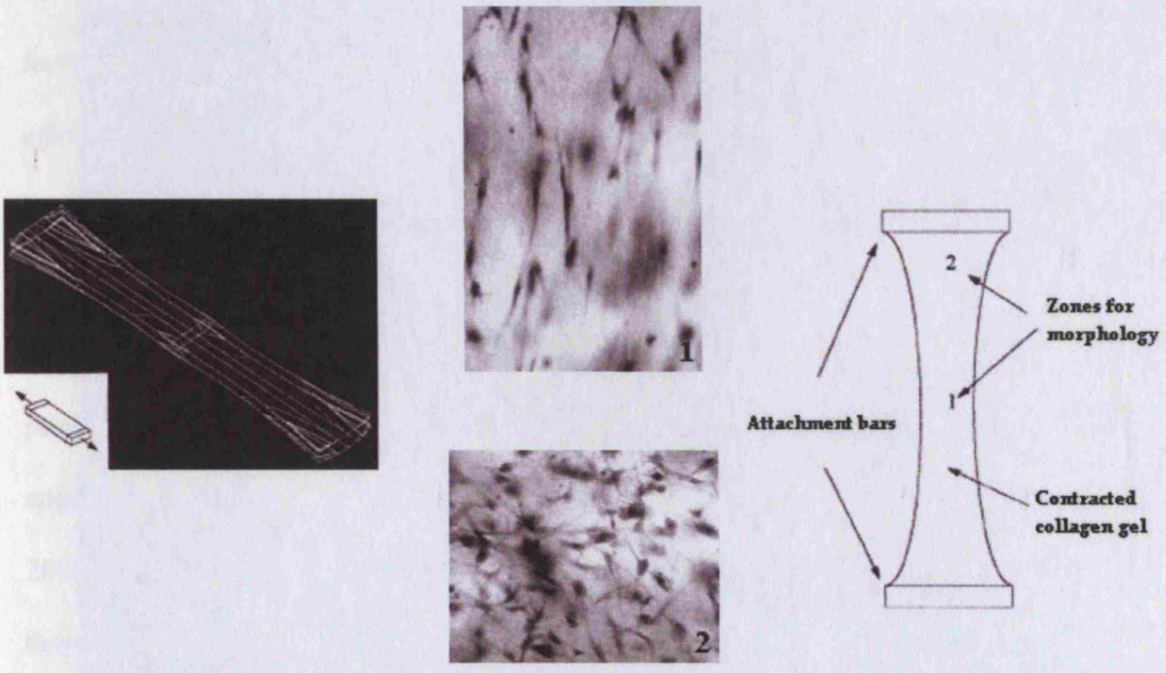


Figure 46: The CFM system provides unidirectional cellular orientation through alignment of cells along the axis of principal strain. FEA analysis predicted

perceived strains by the cells within the collagen constructs and morphology confirmed a distinct cell alignment in zone 1 (Source: adapted from Eastman *et al.*, 1998).

Cell-generated endogenous force in collagen gels promotes differentiation of myoblasts (Vandenburgh *et al.*, 1988). Cheema *et al.* (2003) showed that where contracting gels were tethered uniaxially (in the CFM) C2C12 myoblasts become aligned parallel with the long axis of the gel. The CFM system provides additional cues to guide myoblasts in a particular direction, as uniaxial endogenous tension is generated. As cells attach to the collagen scaffold fibrils, they generate quantifiable contractile forces (measured by the CFM) which depend on cell type, cell attachment, cell density growth factors, and matrix stiffness (Karamichos *et al.*, 2007). *The first aim of this study was to use the CFM to quantify the cell generated endogenous force as the heterogenous mixture of primary human MDCs were seeded in constructs at increasing cell densities (20, 30 and 40 million cells/5ml collagen gel), to test the effect of increasing cell density on force generation and ECM remodeling.*

The use of an optimal design incorporating tissue engineering principles on the one hand and an understanding of the principles involved in the differentiation process of primary myoblasts to oriented functional myofibres on the other hand may yield an approach for the repair of damaged and diseased skeletal muscle in future (Bach *et al.*, 2003). In *in vitro* culture, myoblasts begin to differentiate as a function of their density and the extent of cell-to-cell contact (Gurdon *et al.*, 1993; Cheema, 2004; Pedrotty *et al.*, 2005). Known factors influencing myoblast differentiation include:

- High density of myoblasts
- The presence of extracellular matrix

- Application of mechanical cues and
- Reduction of serum level within the media i.e. depletion of serum factors (Cheema, 2004).

The hypothesis of this study was that increasing cell density will result in increased incidence of cell-to-cell contact and therefore increased myogenic cell differentiation resulting in an up-regulation of myogenin mRNA.

As previously discussed, skeletal muscle is able to adapt its fiber type profile to physiological and functional demands and that this adaptation is reflected in the composition and level of expression of MYHC isoforms (Agbulut *et al.*, 2003). Further, the 'community effect' states that cell interaction is necessary for the activation of certain genes, i.e. large numbers of cells cooperate to become differentiated cells (Gurdon *et al.*, 1993). *The second aim of this study was to test for any transition in the level of MYHC (-IIX/D fast and -β/slow) mRNA expression with increasing cell density in a 3D matrix to determine if the 'community effect' influences muscle fiber phenotype.*

Matrix remodeling by resident cells is an important area of study and MMP-2 is one of the enzymes that remodels the ECM. By regulating the integrity and composition of the ECM in skeletal muscle, MMP-2 plays essential roles in myofiber proliferation, differentiation, fibre healing after injury, and maintenance of the surrounding connective tissue (Carmeli *et al.*, 2004). Knowledge of how the ECM molecules are arranged, remodeled, and interact with the contractile apparatus of craniofacial muscle is of particular interest to those involved in diagnosing and managing craniofacial anomalies (Lewis *et al.*, 2001). The third aim of this study was to test the effect of

increasing cell density on matrix remodeling. *Testing the hypothesis that increasing the cell density would result in increased matrix remodeling and MMP-2 mRNA would be up-regulated as a result.*

The final objective of this study was to allow further maturation of the high density muscle model by culturing the cells for an extended period in 3D culture; enhanced differentiation of the myoblasts to form myotubes was expected as found in other concurrent studies (Vandenburgh, 1991; Dennis *et al.*, 2001; Powell *et al.*, 2002; Cheema *et al.*, 2003). Cheema *et al.* (2003) demonstrated that when C2C12 myoblasts seeded at a high density of 4 million/ml of collagen gel, over a period of 5 days led to optimum differentiation; some degree of development of the 3-D culture (in terms of fusion and maturation of cells to form aligned myotubes) was promoted using static uniaxial loading along the long axis of the culture. This also promoted cell elongation and parallel alignment of myoblasts at high cell density, and myoblast fusion to form multinucleated myotubes over a period of 5–6 days (Cheema *et al.*, 2003). Okano and Matsuda (1998) similarly cultured their "highly dense (cell density 30 million cells/ml), highly oriented hybrid muscular tissues" for 7 days (first for 3 days in a growth medium and then for 4 days in a differentiation medium). Bach *et al.* (2003) emphasized the importance of time-dependent dimensional changes, with respect to the differentiation of myoblasts within a three dimensional matrix. Reporting that the myoblasts cultured in three-dimensional mono-cultures (3 million cells/ml) stained positively for myogenin after 7 days in 3D muscle-neuronal cultures (Bach *et al.*, 2003). The final aim of this study was to culture the 3D constructs for 6 days. *To test the hypothesis that extended time in culture will result in maturation of the constructs and increased mRNA expression of the muscle specific regulatory*

factor (myogenin) and both muscle specific proteins (MYHC-IIX/D and MYHC-β/slow). MMP-2 (marker of ECM remodeling) mRNA was also hypothesised to be up-regulated as the cells continued to remodel the matrix over the 7 day period.

Materials and Methods

The heterogenous mixture of primary human MDCs were cultured as previously described and seeded at increasing concentrations (20, 30 and 40 million cells) into a 5ml collagen gel. The gel was then tethered to the CFM to quantify the endogenous force generated over 24 hours.

In the second part of this study, cells were seeded at the highest cell density of 40 million/5ml collagen gel and matured for 6 days (Figure 47). Serum levels in the media were dropped from 20% to 2% FCS, at days 2 and 5, to promote myogenic cell differentiation.

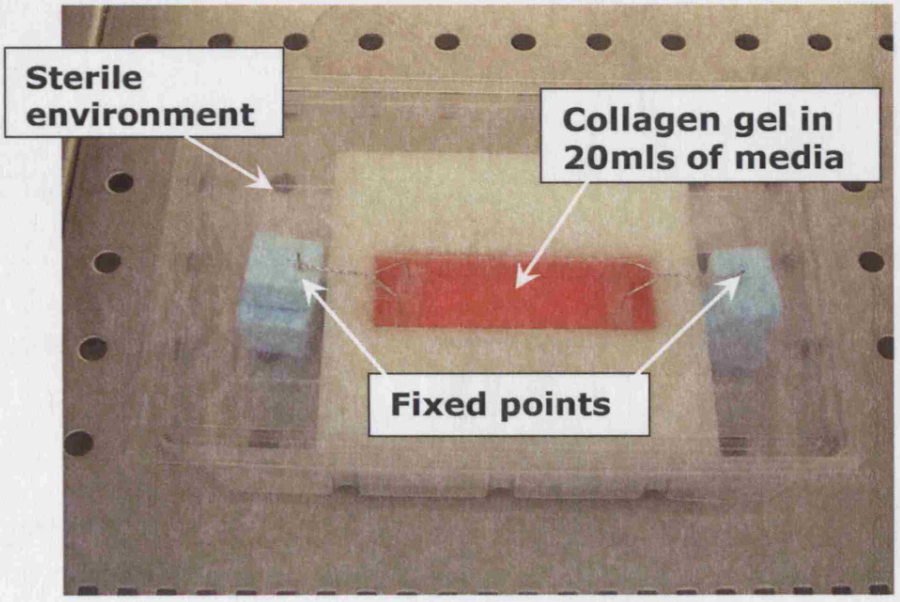


Figure 47: Cells were seeded at a density of 40 million/5ml collagen gel and tethered between two fixed points in a closed, sterile environment for 6 days. The media was changed from normal media (20% FCS) to differentiation media (2% FCS) on day 2, and then another change on day 5, to promote differentiation of myoblasts to form myotubes in 3D collagen lattices.

The RNA was extracted from these gels as previously described and was used in the subsequent cDNA synthesis. PCR was used to amplify the cDNA and data was analysed using the comparative $2^{-\Delta\Delta Ct}$ method.

Results

The endogenous forces generated as cells seeded at high densities remodeled the 3D collagen gel were quantified by the CFM and the resulting force contraction profiles obtained are shown in Figure 48.

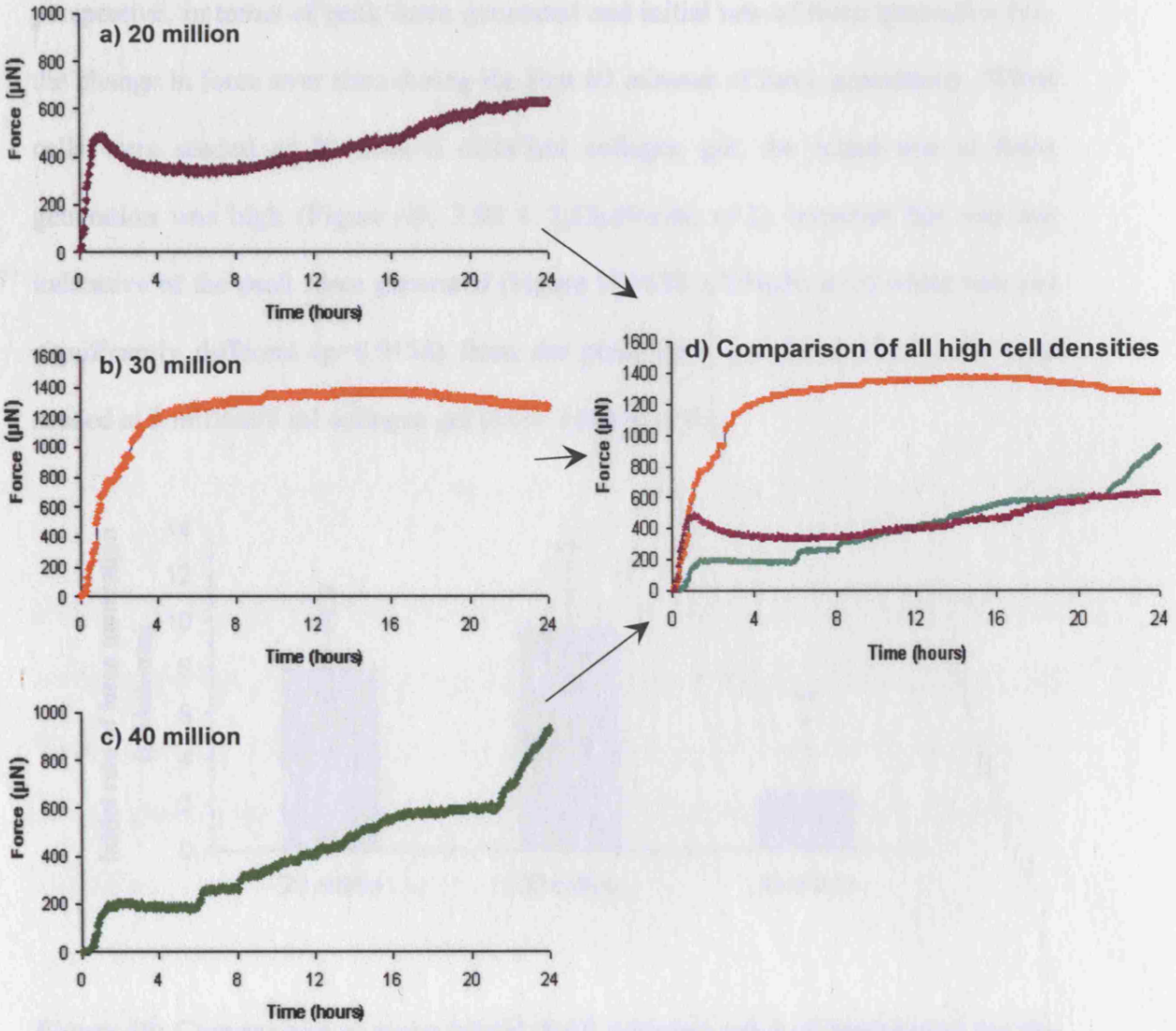


Figure 48: These force contraction profiles were obtained by seeding cells at high densities ((a) 20 million; purple, (b) 30 million; orange, and (c) 40 million; green) into a 5mL collagen gel (n=3). Immediately following gelling, the contraction of the collagen matrix by the resident cells was measured using the

CFM instrument over a 24 hour period. Comparison of the force contraction profiles (d) shows the substantial difference in force generation when cells were seeded at 30 million/5ml collagen gel.

The force contraction profiles obtained were analyzed from a cytomechanical perspective, in terms of peak force generated and initial rate of force generation (i.e. the change in force over time during the first 60 minutes of force generation). When cells were seeded at 20 million cells/5ml collagen gel, the initial rate of force generation was high (Figure 49; 7.92 ± 2.53μN/min; n=3), however this was not indicative of the peak force generated (Figure 50; 622 ± 254μN; n=3) which was not significantly different (p=0.9158) from the peak force generated when cells were seeded at 5 million/5 ml collagen gel (615± 155μN; n=3).

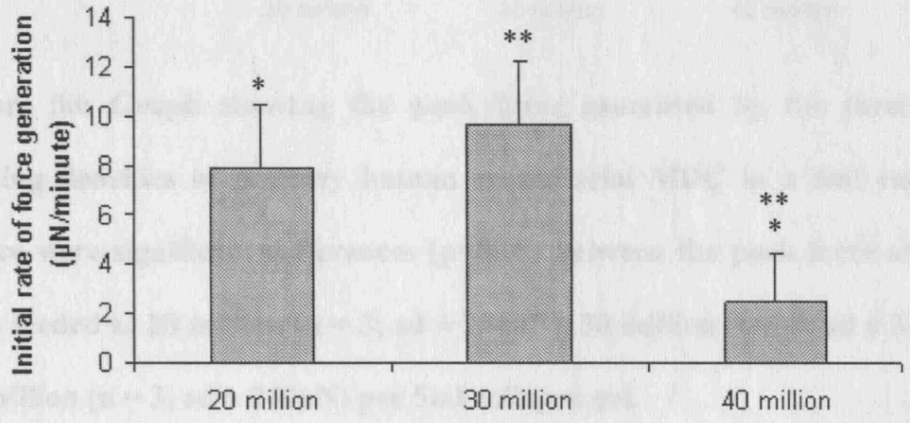


Figure 49: Comparison of mean initial (0-60 minutes) rates of contraction for the three seeding densities. The initial rate of force generation is shown as cells seeded at 20 million (n = 3; sd 7.92 ± 2.53μN/min), 30 million (n = 4; sd 9.58 ± 2.55μN/min) and 40 million (n = 3; sd 2.50 ± 1.90μN/min) contracted the 3D

collagen gel (5ml). Asterix's indicate comparable groups and statistical significance (* $p<0.05$; ** $p<0.01$).

When cells were seeded at 30 million/5ml collagen gel, there was a significant ($p<0.05$) increase in peak force generated with the maximal force of 1373μN (Figure 50; sd ± 313μN; n=4) recorded. Similarly, at this density the rate at which the cells generated force was the highest (9.58 ± 2.55μN/min; n=4).

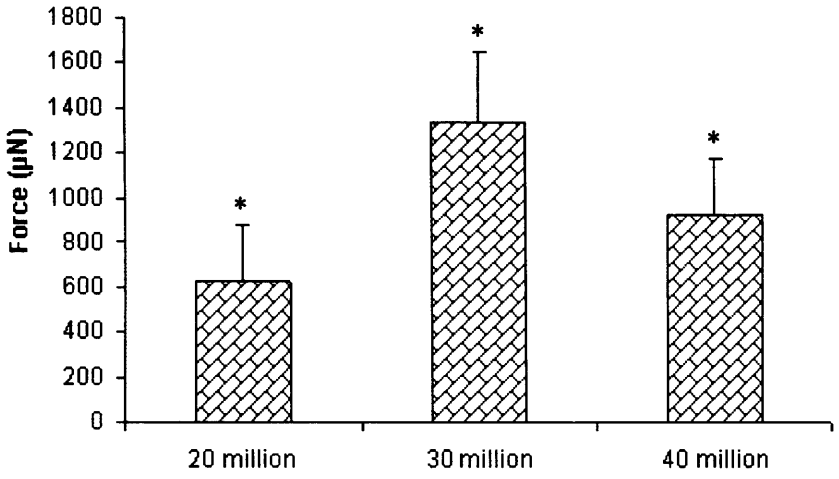


Figure 50: Graph showing the peak force generated by the three different seeding densities of primary human craniofacial MDC in a 5ml collagen gel. There were significant differences ($p<0.05$) between the peak force attained for cells seeded at 20 million (n = 3; sd ± 254μN), 30 million (n = 3; sd ± 313μN) and 40 million (n = 3; sd ± 249μN) per 5ml collagen gel.

The initial rate of force generation (Figure 49; 2.50 ± 1.90μN/min; n=3) when cells were seeded at 40 million/5ml construct was significantly lower than the rate recorded for 20 and 30 million ($p<0.05$ and $p<0.01$ respectively) and the peak force (Figure 50; 926 ± 249μN; n=3) generated was significantly different ($p<0.05$) and fell between the peak forces generated when cells were seeded at 20 and 30 million. However,

there was a distinctive pattern of force generation, not reflecting the characteristic three phases of traction, contraction and force plateau, but rather a continual incremental increase of force generation with time.

From this cytomechanical analysis, it was clear that the optimal cell seeding density, with respect to maximal peak force and rate of initial force generation, is 30 million cells/5ml construct. However, the distinctive force contraction profile obtained when cells were seeded at 40 million/5ml construct is indicative of cell-to-cell interaction generating a gradual, but continual increase in force over time.

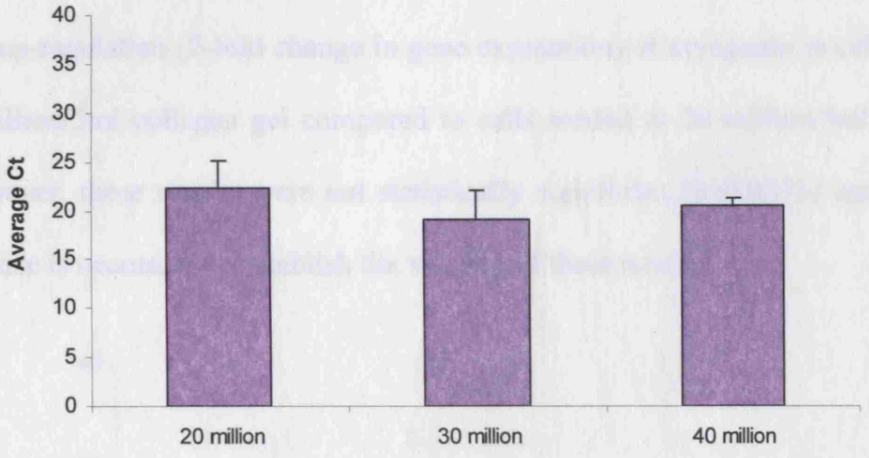


Figure 51: The effect of different cell seeding densities (per 5ml collagen gel) on the expression of the housekeeping gene GAPDH. Standard deviation was used for error bars. There was no significant difference (p=0.3292) in gene expression so GAPDH was used as the internal control in this study.

As previously stated, using the $2^{-\Delta\Delta Ct}$ method, the data are presented as the fold change in gene expression normalized to an endogenous reference (GAPDH) and relative to a control (normal 5 million cells/5ml collagen gel). It was again important

to establish that the internal control gene was properly validated for each experiment to determine that gene expression is unaffected by the experimental treatment (Livak and Schmittgen, 2001). Figure 51 shows the expression of GAPDH at each cell density. No significant difference (p=0.3292) was found in GAPDH expression between experimental conditions. Therefore GAPDH was verified as an appropriate housekeeping gene in this study.

To establish that there was evidence of myogenic cell differentiation within the 3D constructs, Figure 52 revealed that after 24 hours in culture and at all three high cell densities, the 3D constructs expressed myogenin mRNA. There was an apparent relative up-regulation (7-fold change in gene expression) of myogenin in cells seeded at 30 million/5ml collagen gel compared to cells seeded at 20 million/5ml collagen gel, however, these results were not statistically significant (p=0.8375) and a larger sample size is necessary to establish the validity of these results.

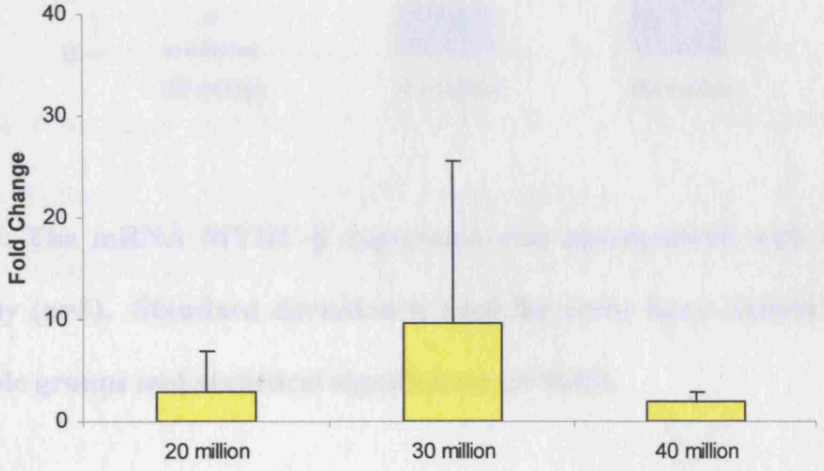


Figure 52: Myogenin gene expression is shown for MDCs seeded at increasing cell densities per 5ml collagen gels (n=3). Standard deviation is used for error bars.

The aim of the next part of the study was to examine any transitions with increasing cell seeding density and determine the relative composition of the two MYHC isoforms; the fast isoform (MYHC-IIX/D) and the slow isoform (MYH-β). Figure 53 shows the increasing gene expression of MYHC-β with cell density. There was an 8 fold increase when MDCs were seeded at 30 million/5ml collagen gel and a 40 fold increase when cells were seeded at 40 million/5ml collagen gel. These results were significantly different ($p<0.05$) by One-way ANOVA.

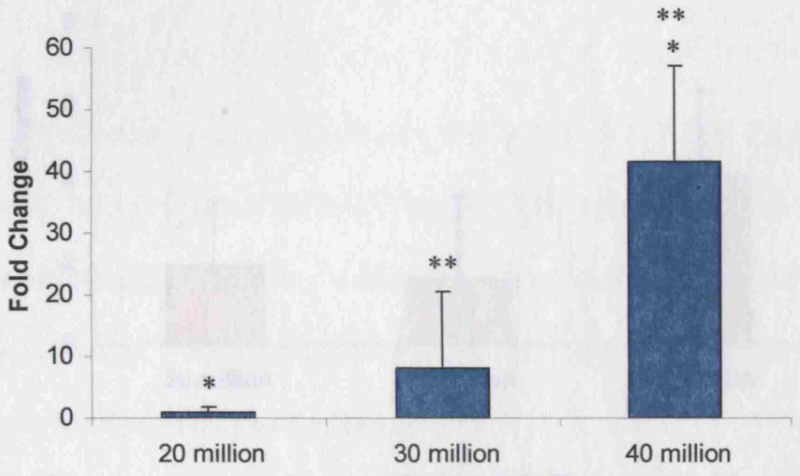


Figure 53: The mRNA MYHC-β expression was up-regulated with increasing cell density (n=3). Standard deviation is used for error bars. Asterix's indicate comparable groups and statistical significance ($p<0.05$).

One-way ANOVA revealed no significant difference (p=0.4809) in MYH-IIX/D gene expression with increasing cell density. As depicted in Figure 54 the relative fold change between the 20 million and 30 million/5ml construct was negligible, 1.95 and 1.63 respectively. There was a trend suggesting a potential up-regulation of MYHC-IIX/D when MDCs were seeded at 40 million/5ml collagen gel with a relative fold increase of 4.16, more than double the amount present at lower densities, however, these results were not statistically significant and a larger sample size should be investigated.

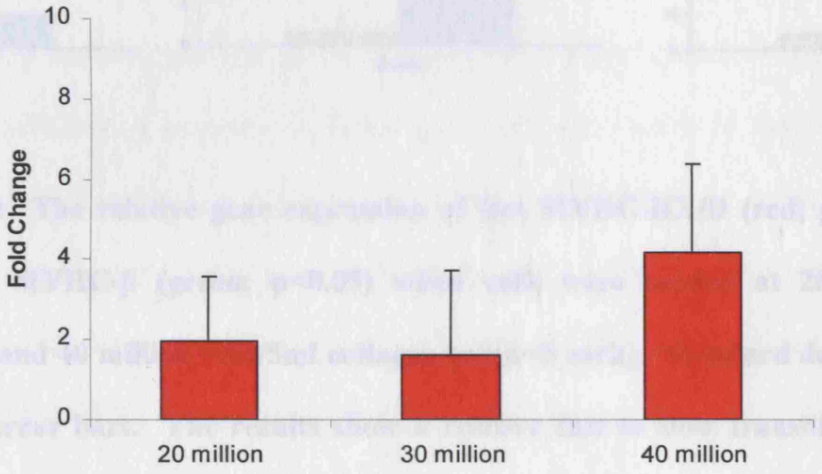


Figure 54: The gene expression of MYHC-IIX/D varied with increasing cell density (n=3 each). Standard deviation is used for error bars. There was no significant difference (p=0.4809) in mRNA expression between the groups.

To examine any transitions with increasing cell seeding density the relative expression of MYHC-IIX/D and MYHC-β were plotted at each cell seeding density (Figure 55). The results suggest a transition with a relative switch from fast to slow MYHC isogene expression with increasing cell density indicative of a change in phenotype as a result of 'community effect'.

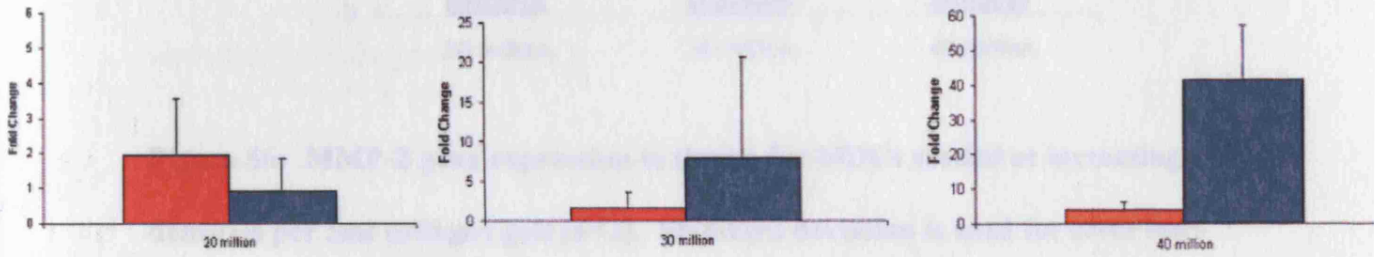


Figure 55: The relative gene expression of fast MYHC-IIX/D (red; p=0.4809) and slow MYHC-β (green; p<0.05) when cells were seeded at 20 million, 30million and 40 million cells/5ml collagen gel (n=3 each). Standard deviation is used for error bars. The results show a relative fast to slow transition of the isogene expression with increasing cell density.

The final gene expression presented in this study is the matrix metalloproteinase-2 (MMP-2) enzyme; one of the enzymes that remodels the ECM. The results in Figure 56 show an up-regulation of MMP2 mRNA (3-fold change) when MDCs were seeded at 40 million/5ml gel compared to when cells were seeded at 20 million/5ml gel. However, due to the lack of statistical significance (p=0.3932), variations in these results are only suggestive of a possible trend and again, a larger sample size should be considered.

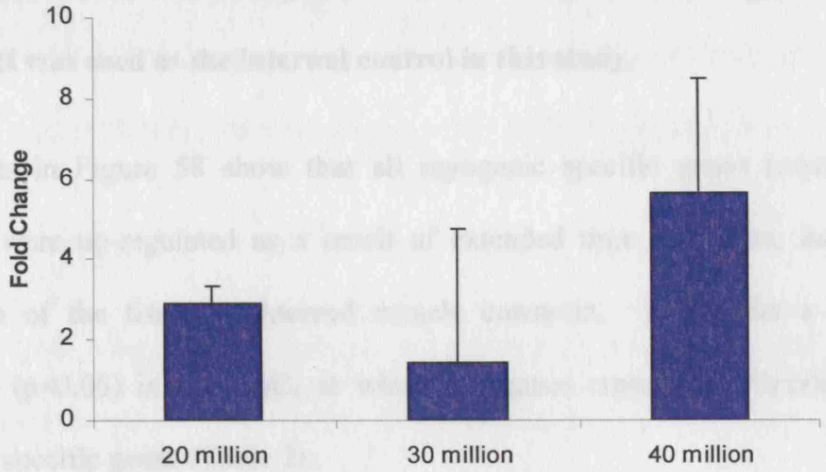


Figure 56: MMP-2 gene expression is shown for MDCs seeded at increasing cell densities per 5ml collagen gels (n=3). Standard deviation is used for error bars

Having established expression of these genes after 24 hours in culture, it was hypothesized that maturation of these constructs would result in an up-regulation of all the associated genes. Cells were seeded at 40 million/5ml construct and cultured for 6 days. RNA was extracted and processed for PCR. After 6 days in culture expression of GAPDH was not significantly different (p=0.2421).

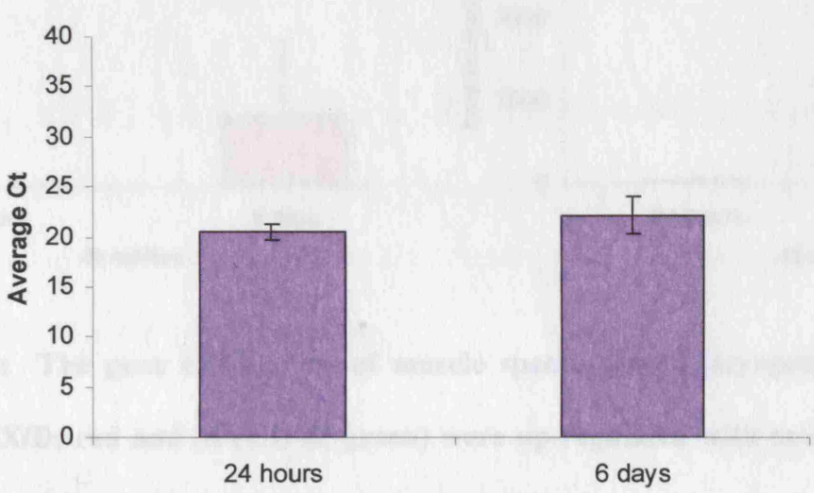


Figure 57: Expression of the housekeeping gene GAPDH after 24 hours and after an extended period (6 days) of culture when cells were seeded at 40 million/5ml

collagen gel. There was no significant difference (p=0.2421) in gene expression so GAPDH was used as the internal control in this study.

The results in Figure 58 show that all myogenic specific genes (myogenin and MYHCs) were up-regulated as a result of extended time in culture, indicative of maturation of the tissue engineered muscle construct. There was a significant difference ($p<0.05$) in the $\Delta\Delta C_t$ at which the genes crossed the threshold for all myogenic specific genes (Table 2).

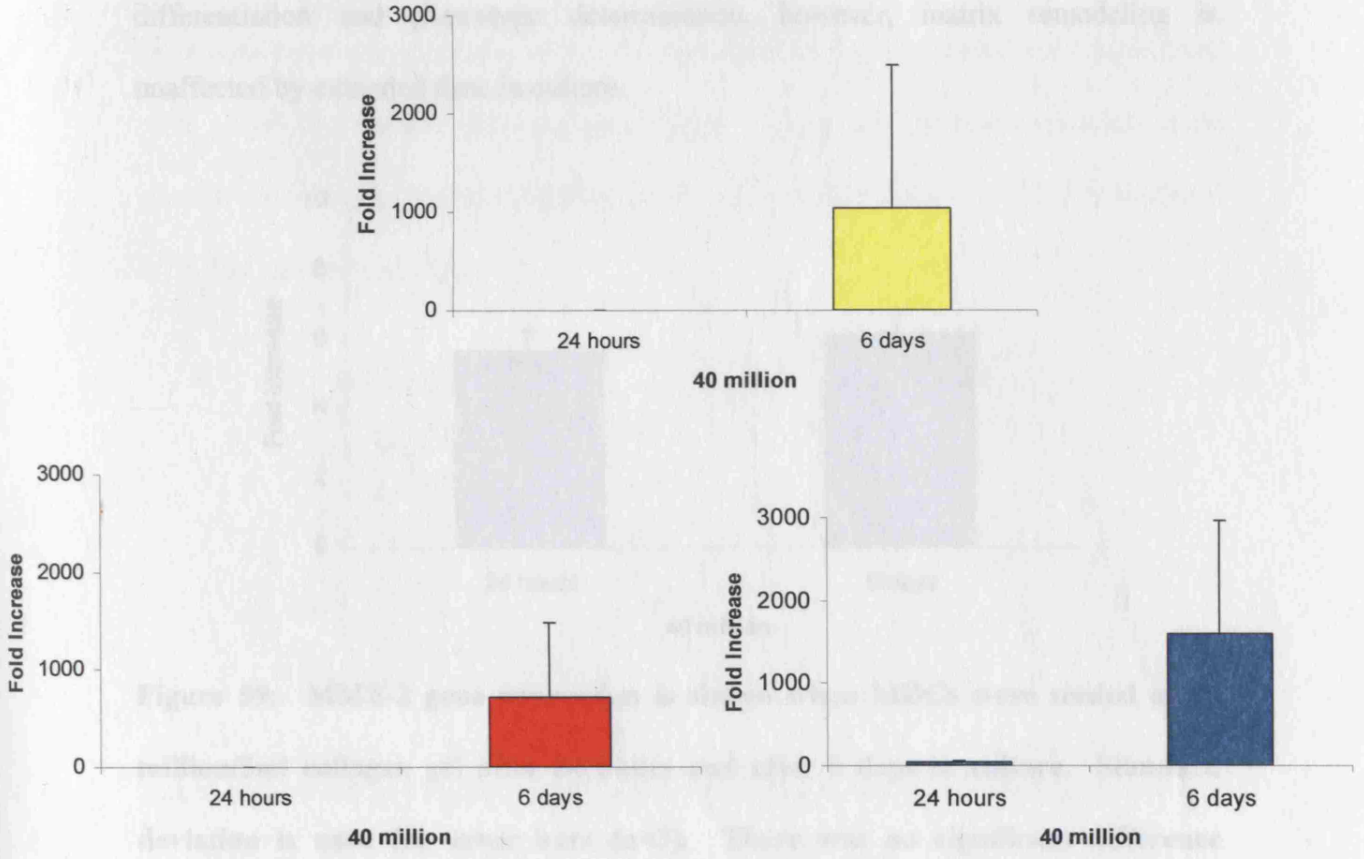


Figure 58: The gene expressions of muscle specific genes (myogenin; yellow, MYHC-IIX/D; red and MYCH-β; green) were up-regulated with extended time in culture. Standard deviation was used for error bars (n=3).

However, the actual fold change gene expression (myogenin, 1038; MYHC-IIX/D, 715; and MYCH-β. 1590) was not significantly (myogenin, p=0.2847; MYHC-IIX/D, p=0.3061; and MYCH-β p=0.1201 respectively) different for the number of samples evaluated.

Finally, the fold change gene expression of MMP-2 did not significantly (p=0.8354) differ between 24 hours (1.24 ± 0.63; n=3) and 6 days (1.36 ± 0.70; n=3) in culture. This would suggest that construct maturation occurs in terms of myogenic cell differentiation and phenotype determination, however, matrix remodeling is unaffected by extended time in culture.

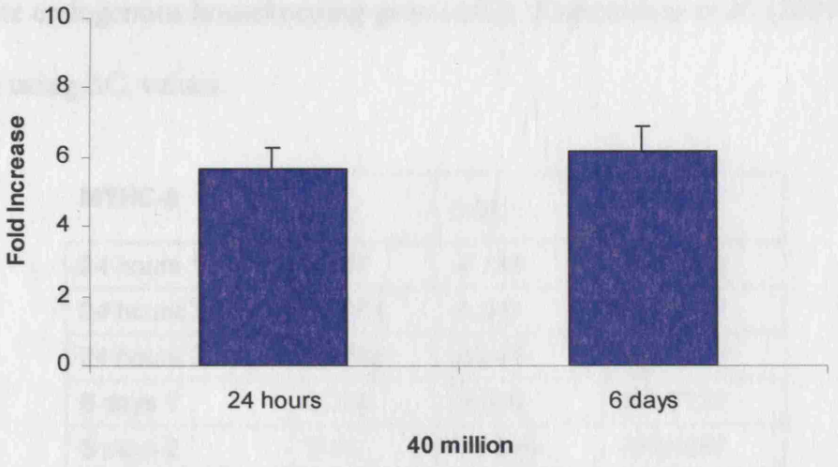


Figure 59: MMP-2 gene expression is shown when MDCs were seeded at 40 million/5ml collagen gel after 24 hours and after 6 days in culture. Standard deviation is used for error bars (n=3). There was no significant difference (p=0.8354) in MMP-2 gene expression.

A note on statistical analysis of RT-PCR data

The endpoint of real-time PCR analysis is the threshold cycle or C_t. In order to relatively quantify gene up-regulation/down-regulation Karamichos *et al.* (2007) compared the Threshold Cycle (C_t) values of the samples of interest with a control or calibrator such as a non-treated sample. The C_t reflects the cycle number at which the fluorescence generated within a reaction crosses the threshold. The C_t assigned to a particular sample reflects the point during the reaction at which a sufficient number of amplicons have accumulated, within the well/sample, to be a statistically significant point above the baseline (Karamichos, 2006). The C_t values are normalized to an appropriate endogenous housekeeping gene (ΔC_t). Karamichos *et al.* (2007) analysed PCR data using ΔC_t values.

MYHC-β	ΔC_T	$\Delta\Delta C_T$	$2^{-\Delta\Delta Ct}$
24 hours 1	11.88	-4.728	26.50146
24 hours 2	11.261	-5.347	40.70122
24 hours 3	10.762	-5.846	57.52033
6 days 1	7.779	-8.829	454.7721
6 days 2	5.01	-11.598	3099.887
6 days 3	6.59	-10.249	1216.904

Table 6: The endpoint of RT-PCR analysis PCR analysis is the threshold cycle or C_t. The raw data for MYHC-β gene expression after 24 hours (n=3) and after 6 days (n=3) is summarised in this table. The ΔC_t is normalization to the internal housekeeping gene GAPDH. $\Delta\Delta C_t$ is relative to an external control. The C_t values are exponential and not linear terms, and it is necessary to convert the data into a linear term using the comparative $2^{-\Delta\Delta Ct}$ method for statistical significance.

However, Livak and Schmittgen (2001) gave an extensive report in which they highlighted that the C_t is determined from a log–linear plot of the PCR signal versus the cycle number. Thus, C_t is an exponential and not a linear term. For this reason, any statistical presentation using the raw C_t values should be avoided (Livak and Schmittgen, 2001). Presentation of relative PCR data is most often calculated along with an internal control and/or calibrator sample and is rarely presented as the C_t, an exception is when one is interested in examining the sample-to-sample variation among replicate reactions, e.g. expression of a housekeeping gene (Livak and Schmittgen, 2001). *Statistical data should be converted to the linear form by the* $2^{-\Delta\Delta Ct}$ *calculation and should not be presented by the raw* C_T *values* (Livak and Schmittgen, 2001).

	$\Delta\Delta C_T$	**Fold Change**
Myogenin	Significant ($p<0.05$)	Not significant ($p=0.2847$)
MYHC-IIX/D	Significant ($p<0.05$)	Not significant ($p=0.3061$)
MYHC-β	Significant ($p<0.05$)	Not significant ($p=0.1201$)

Table 7: Statistical analysis of $\Delta\Delta C_t$ vs. fold change ($2^{-\Delta\Delta Ct}$) for myogenic specific genes. The difference in ΔΔCt was statistically significant ($p<0.05$). However, the actual fold change ($2^{-\Delta\Delta Ct}$) gene expression were not significantly different.

Table 1 shows the raw data for MYHC-β gene expression after 24 hours (n=3) and after 6 days (n=3). Notice the small variation in the values of the exponential terms ΔC_t and $\Delta\Delta C_t$, these values are statistically different (Table 2; $p<0.05$). However, due

to the nature of exponential amplification, when these values are converted to the linear form ($2^{-\Delta\Delta Ct}$) as a true representation of the *fold change in gene expression*, the values are not significantly different (Table 2). In this study, the lack of statistical significance can be explained as a consequence of converting the results of an exponential process into a linear comparison of amounts (Livak and Schmittgen, 2001).

Discussion

In vitro tissue engineering of skeletal muscle involves culturing myogenic cells in an environment that emulates the 3D *in vivo* environment so that the cells proliferate, fuse, organize in three dimensions, and differentiate into functional skeletal muscle capable of generating force (Kosnik *et al.*, 2003). The development of 3D skeletal muscle constructs has made possible the measurement of contractile properties of cultured skeletal muscle cells (Dennis and Kosnik, 2000). In this study the force contraction profiles obtained were distinctly different at each cell seeding density. When cells were seeded at 20 million/5ml collagen gel, the contraction profile had the characteristic phases of traction, contraction and force plateau and the peak force generated was not significantly different from when cells were seeded at 5 million/5ml collagen gel (Chapter 2).

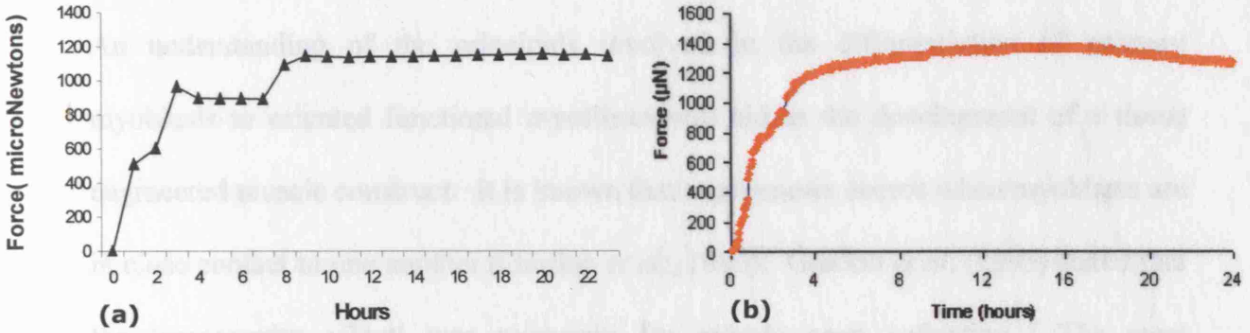


Figure 60: Comparison of the published 'typical myotube contraction curve' (Cheema *et al.*, 2003) with the contraction profile obtained when primary human MDCs were seeded at 30 million/5ml collagen gel.

The force contraction profile obtained when cells were seeded at 30 million/5ml collagen gel was comparable (Figure 60) to the 'typical myotube contraction curve'

published by Cheema *et al.* (2003). Cheema *et al.* (2003) attempted to tissue engineer a differentiated skeletal muscle construct using C2C12 mouse skeletal myoblasts (seeded at 20 million cells/5ml collagen gel) and monitored the isometric contractile properties of the myotube-rich collagen gels. The peak force generated by the C2C12 neo-muscle organoid was 1150μN (Cheema *et al*, 2003), the peak force generated by the primary human MDC neo-muscle organoid in this study was 1373μN. Further, the initial rapid rate of force generation/contraction was similar. Force generation for myotube gels was characterised by a series of rapid contractions, or jumps, which may represent a form of synchronous myotube contraction (Cheema *et al.*, 2003). The distinctive pattern of force generation when cells were seeded at 40 million/5ml collagen gel, with incremental increases in force generation with time may have resulted from the complex interplay between cell-cell and cell-matrix interactions of myogenic cells.

An understanding of the principals involved in the differentiation of primary myoblasts to oriented functional myofibres will aid in the development of a tissue engineered muscle construct. It is known that myogenesis occurs when myoblasts are in close contact to one another (Gurdon *et al.*, 1993). Gurdon *et al.* (1993) stated that the 'community effect' was necessary for muscle gene activation. The most important feature of the 'community effect' is that a large number of cells are required for a muscle response (Gurdon *et al.*, 1993). Myoblast fusion, by which mononucleated myoblasts fuse to form multinucleated muscle fibers, is an essential early step during skeletal muscle differentiation (Chen and Olsen, 2004). Myoblasts recognize one-another, adhere and align to form linear aggregates, and then the cells

of these aggregates fuse to form a myotube that will mature to a muscle fibre (Clark *et al.*, 2002).

The fusion of mononucleate precursor myoblasts to form the multiucleated skeletal muscle fibre is preceded by a series of complex cell-cell interactions (Knusden, 1990; Dickson *et al.*, 1990). Many changes that accompany fusion have been described. For example, during myogenesis *in vivo* and *in vitro*, expression of the neural cell adhesion molecule (NCAM) undergoes an isoform transition that precisely correlates with terminal myoblasts differentiation and myotube formation (Dickson *et al.*, 1990; Charlton *et al.*, 2000). Another change accompanying skeletal muscle cell fusion is a dramatic increase in synthesis of muscle specific proteins, e.g. myosin heavy chain (Wiid *et al.*, 1984).

Further, it is generally agreed that for membrane fusion to occur, a fluid membrane state is needed and previous studies have shown that when cells fuse, the greatest changes in membrane fluidity occur in areas of cell contact between fusing cells (Herman and Fernandez, 1978, 1982). This increase in membrane fluidity results from the redistribution of surface antigens (Kaufman and Foster, 1985) and changes in integrin expression (Clark *et al.*, 1997). The fusion of membranes also involves changes in the lipid structure of the bilayers during the process (Wakelam & Pette, 1982). It is likely that together, many of these events favour strong cell–cell adhesion over cell–substratum adhesion, enabling the myoblasts to assume the correct shape and geometrical arrangement for fusion (Clark *et al.*, 2002).

During the terminal stage of skeletal myogenesis, myoblasts stop replicating, fuse to form multinucleate fibres, and express the genes that encode the proteins (MYHC) that convey contractile capacity (Kaufman and Foster, 1988). The results of this study showed that there was a significant increase in MYHC-β with increasing primary human MDC density in 3D collagen gels. Indicative of increased terminal differentiation of myogenic cells within the muscle constructs, with increasing cell density. However, the gene expression of myogenin and MYHC-IIXD did not significantly differ, nor follow the trend of increased expression with increasing cell density. There was also an apparent transition in the fast-to-slow direction of MYHC isogene expression with increasing cell density, suggesting that the 'community effect' influences the determination of muscle fiber type.

It is axiomatic that for cells to bring about 'contraction' of a collagen gel, it is essential for them to attach to the matrix or to each other. Although cell-matrix adhesion is of importance for the progression of myogenesis, it is not directly involved in myotube formation. In the early stages of myoblast differentiation, when cells become postmitotic but have yet to fuse, cell–cell adhesion is probably more important than cell–matrix adhesion for efficient cell fusion (Wakelam, 1985; Clark *et al.*, 1997). A switch from cell–matrix adhesion to cell–cell adhesion could explain why after 6 days in 3D culture the skeletal muscle specific factors (myogenin) and protein (MYHC) mRNA were upregulated, but MMP-2 matrix remodeling remained constant.

Conclusions

- In this study, cell generated endogenous forces as primary human MDCs were seeded in constructs at increasing cell densities were quantified. Establishing that the optimal cell seeding density for peak force generation and ECM remodeling (initial rate of force generation) was 30 million cells/5 ml collagen gel.

- The hypothesis that increasing cell density would result in increased incidence of cell-to-cell contact and therefore more myogenic cell differentiation, resulting in a linear upregulation of myogenin mRNA proved to be an oversimplification of myogenic differentiation in 3D constructs at high cell densities. There was no significant up-regulation of myogenin mRNA as cell density increased.

- The relative expression and switching of MYHC-IIX/D (fast) and MYHC–β (slow) mRNA expression was studied in response to increasing cellular density within a tissue engineered 3D skeletal muscle model. There was a significant increase of MYHC–β mRNA expression with increasing cell density. However, there was no significant change in MYHC-IIX/D mRNA gene expression.

- MMP-2 was not significantly up-regulated with increasing cell density. Therefore, increasing cell density does not result in increased matrix remodeling.

- Extended time in culture resulted in an up-regulation of mRNA expression of the muscle specific regulatory factor (myogenin) and the muscle specific proteins (MYHC-IIX/D and MYHC-β/slow), indicative of maturation of the constructs by an increase in myogenic differentiation and fibre phenotype determination. However, MMP-2 mRNA expression, and ultimately matrix remodeling, did not alter significantly over the 7 day period.

Ch 5: MDC response to increased matrix stiffness

Introduction

The native extracellular matrix (ECM) acts as a scaffolding for cells in a tissue, gives it structure, and regulates cell phenotype (e.g., tissue-specific gene expression) (Rosso *et al.*, 2004). Tissue engineering approaches typically employ an exogenous 3D matrix to simulate the native ECM and engineer new tissues from native cells (Abatangelo et al., 2001). The exogenous matrices are designed to provide an appropriate 3D environment for the cells, mechanical strength and structural stability, until the newly formed tissues are mechanically stabilized and specific signals occur to guide the gene expression of cells forming the tissue (Putnam and Mooney, 1996).

Numerous forms of scaffold materials are currently under investigation and the matrices used in tissue engineering applications are divided into synthetic and biologically-derived biomaterials (Freed *et al.*, 1994; Grande *et al.*, 1997; Cronin *et al.*, 2004; Bach *et al.*, 2004). Naturally derived biopolymers have been shown to possess potentially excellent characteristics as templates for tissue regeneration (Bitar *et al.*, 2007). Collagen, fibronectin, chitosan, starch, and hyaluronic acid are examples of the many native biopolymers currently in use. These biopolymers, whether animal or plant in origin, have attracted much interest due to their low immunogenic potential and the capacity of integrating with the host tissue (Novikova *et al.*, 2003).

Collagen directly supports integrin-mediated cell attachment and the subsequent expression, and maintenance, of the seeded cell differentiated state (Bitar *et al.*,

2007). One of the main advantages of collagen as a scaffold is its properties as a cell substrate; it is biocompatible, has low immunogenicity, is conserved across species, and is naturally remodelled by cells (Abou Neel *et al.*, 2006). As the main extracellular matrix (ECM) protein in musculoskeletal tissues, collagen has been the scaffold material of choice throughout this thesis. However, owing to an extremely low protein to water ratio, these hydrated matrices have no inherent mechanical strength thus limiting their immediate *in vivo* application (Giraud Guille *et al.*, 2005; Abou Neel *et al.*, 2006; Bitar *et al.*, 2007; Karamichos *et al.*, 2007). This is true for many engineered tissues, in which the mechanical properties remain inferior to those of native tissues (Cao *et al.*, 1994; Kim *et al.*, 1999; Mauck *et al.*, 2000). This has necessitated the use of various methods to enhance the mechanical properties and integrity of such constructs. Several investigators have attempted to improve the functionality of engineered tissues by mimicking the *in vivo* environment through application of external mechanical load to tissues *in vitro* (Kim *et al.*, 1999; Mauck *et al.*, 2000; Banes *et al.*, 2003; Karamichos *et al.*, 2007).

Nearly 30 years ago Bell *et al.* (1979) reported that fibroblasts condensed a hydrated collagen gel to produce a denser arrangement of collagen fibrils, with water being excluded in the process. The potential of this finding was not realized until Brown *et al.* (2005) developed a novel technique to expel the interstitial fluid from hyperhydrated collagen constructs producing tissue-like collagenous scaffolds of considerable mechanical strength. The process is both simple and rapid, exploiting the central property of the gels wherein compression expels the liquid which does not return on removal of the load, i.e. the gel undergoes a plastic compression (PC) (Brown *et al.*, 2005). PC removes fluid from hyper-hydrated gels in a matter of

minutes to produce scaffolds that are dense, cellular, and mechanically strong, with controllable meso-scale structures (Abou Neel *et al.*, 2006). Bitar *et al.* (2007) showed that plastic compressed constructs were capable of supporting cell viability and proliferation of human dermal fibroblasts for 5 weeks in culture concluding that PC of hydrated collagen gels rapidly produces biomimetic scaffolds of improved mechanical properties and these scaffolds can potentially be utilized as cell seeded systems for tissue engineering (Bitar *et al.*, 2007). *The objective of this study was to produce a stiffer matrix for tissue engineering skeletal muscle and study muscle derived cellular response. The process of plastic compression was utilized and methodology was developed for tethered collagen constructs.*

The ECM plays a vital role in regulating the response of cells to signals from their microenvironment through mediation of cell adhesion and regulation of the cellular force balance (Simmons and Mooney, 2003). The stiffness of the matrix can dictate the fate and function of a cell, Rowley and Mooney (1999) showed that C2C12 skeletal myoblasts cultured on modified hydrogels (RGD-coupled alginates) with high compressive modulus showed increased proliferation and differentiation compared with those on medium and low stiffness gels. Further, the stiffness of the material may regulate the ability of the matrix to resist cell-based tractional forces, thereby mediating changes in the force balance between the cell and matrix, and within the cell (Simmons and Mooney, 2003). *The first aim of this study was to test the effect of a stiffer matrix (using PC) on generation of cell-based tractional forces. This was achieved by measuring the cytomechanical force generated by MDCs embedded in plastic compressed collagen. Testing the hypothesis that a stiffer matrix would result in a reduced generation of quantifiable force.*

As discussed in chapter 2, there is a force balance between the tension generated by the cytoskeleton of a cell and the resistance provided by the cells adhesion to its matrix that determines the shape of the cell (Simmons and Mooney, 2003). Cell shape correlates with several cellular behaviours, including growth, differentiation, apoptosis, motility and signalling (Chen *et al.*, 1997; Ingber, 1990; Mooney *et al.*, 1992) and it has been suggested that the fate of a cell may be regulated by the stiffness of the substrate to which it is adhered (Simmons and Mooney, 2003). *The second aim of this study was to use TEM to qualitatively analyse the effect of plastic compression on cell shape and collagen fibril density.*

Hydrogels enable specific cell-matrix interactions, which has the potential to allow for control of gene expression of cells within the matrices (Simmons and Mooney, 2003). For tissue engineering strategies it is essential to know how cells interact with the ECM and transduce the information received by the extracellular molecules into an intracellular event (Rosso *et al.*, 2004). Further, cell-matrix actions regulate gene expression and ultimately tissue development and function (Streuli, 1999). Because the matrix plays a significant role in regulating tissue development, defining the roles of the matrix and its interaction with cells are critical to designing tissue engineering matrices that present the signals necessary for appropriate gene expression and functional tissue development (Simmons and Mooney, 2003). *The third aim of this study was to quantify the gene expression of the muscle specific factor, myogenin, and the muscle specific proteins, MYHC-IIX/D and MYHC-β using RT-PCR, to determine whether the process of plastic compression promoted the expression of markers of myogenic terminal differentiation by increasing the incidence of cell-to-cell contact in*

the 3D collagen construct. Testing the hypothesis that muscle specific gene expression will be up-regulated in PC constructs.

The ability of the matrix to regulate the cellular microenvironment can be exploited by designing matrices that present the appropriate signals necessary to direct cell function to engineer functional tissues (Simmons and Mooney, 2003). Huang and Ingber (1999) suggested that the ability of cells to respond to the stiffness of their substrate may play a critical role in regulating tissue remodeling. Further, Karamichos *et al.* (2007) suggested that increasing the stiffness of collagen constructs will allow the tailoring of specific cellular responses in tissue engineering applications. *The final aim of this study was to quantitate the effect of increased stiffness (due to PC) on the expression of the matrix remodeling gene MMP-2 in PC MDC seeded constructs. Testing the hypothesis that the stiffer matrix will induce a significant up-regulation of MMP-2 gene expression in primary human MDC seeded constructs.*

Materials and Methods

Methods development

Plastic compression of hydrated collagen gels rapidly produces biomimetic scaffolds of improved mechanical properties (Brown *et al.*, 2005; Abou Neel *et al.*, 2006; Bitar *et al.*, 2007). In this study we developed the methodology for applying the novel process of plastic compression to tethered collagen constructs. This enabled the force contraction profile of a tethered plastic compressed MDC seeded collagen construct to be measured on the CFM.

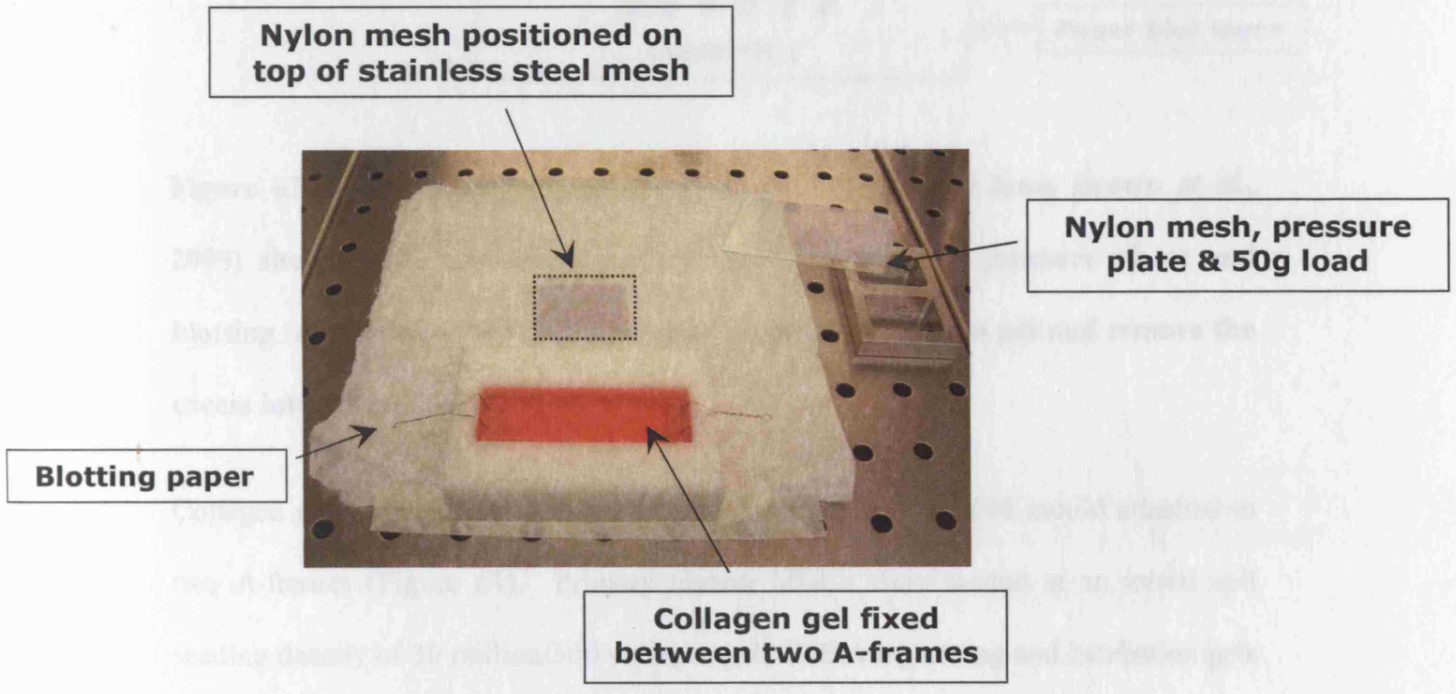


Figure 61: This picture depicts the pre-compressed set up. Collagen gels were prepared as normal in the CFM mould between two A-frames and immersed in 20ml of medium. The blotting paper and meshes (stainless steel and nylon) were positioned in the centre of the blotting paper and the pressure plate and load are also shown before the process of plastic compression.

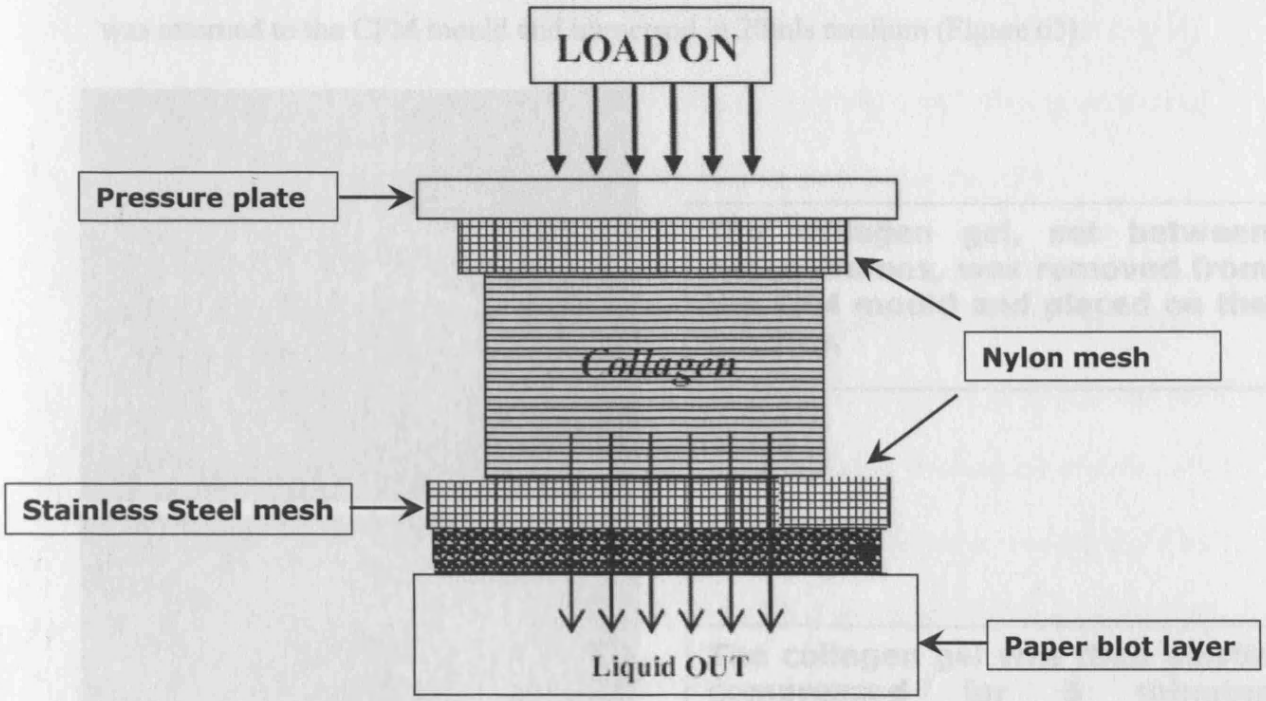
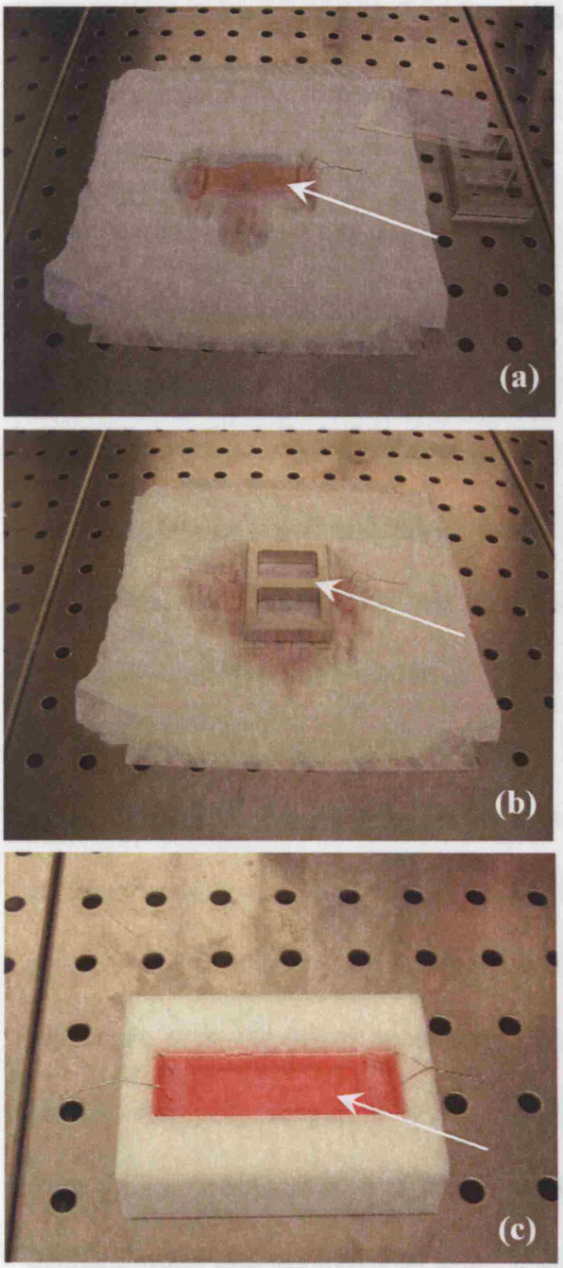


Figure 62: Plastic Compression (PC) schematic (Adapted from Brown *et al.*, 2005) showing the combination of compression (load on pressure plate) and blotting (mesh and paper blot layers) to sandwich a collagen gel and remove the excess interstitial fluid.

Collagen gels were prepared as previously described in the CFM mould attached to two A-frames (Figure 61). Primary human MDCs were seeded at an initial cell seeding density of 30 million/5ml collagen gel. Following setting and incubation gels were routinely compacted by a combination of compression and blotting using layers of mesh and paper sheets as shown in the schematic of Figure 62. A 165µm thick stainless-steel mesh (mesh size ~ 300µm) and a layer of nylon mesh (~50µm; to prevent sticking) were placed on a double layer absorbent paper (Brown *et al.*, 2005). The tethered collagen gel was placed on the nylon mesh, covered with a second nylon mesh, pressure plate and loaded with a 50g flat stainless steel block for 5 min at room

temperature leading to the formation of a flat collagen sheet (~20-40μm thick) which was returned to the CFM mould and immersed in 20mls medium (Figure 63).



The collagen gel, set between two A-frames, was removed from the CFM mould and placed on the meshes.

The collagen gel was then plastic compressed for 5 minutes beneath the pressure plate and 50 g load.

The PC collagen gel was returned to the CFM mould and immersed in medium.

Figure 63: The PC process. The attached collagen gel was carefully removed from the CFM mould and placed on the nylon mesh (a). The second nylon mesh, pressure plate and 50g load were carefully placed on top of the collagen gel and plastic compressed for 5 minutes (b). The plastic compressed collagen sheet was then returned to the CFM mould (c) and immediately attached to the CFM.

The PC collagen sheet was returned to the mould and then tethered to the CFM to quantitate the force contraction profile generated as the cells responded to the PC matrix. After 24 hours the gels were removed from the CFM and RNA was extracted, converted into cDNA and amplified using RT-PCR as previously described.

Transmission Electron Microscopy (TEM) was used to qualitatively analyse the effect of the PC process on MDCs seeded within a collagen gel. TEM is a powerful tool for high magnification and high resolution image analysis giving information of cells and organelles. Specimens of normal and PC collagen constructs were washed in 0.1M phosphate buffer, fixed in 2.5% gluteraldehyde in 0.1M phosphate buffer for 1 hour at 4^0C. This was followed by two phosphate buffer washes and a secondary fixation with 1% osmium tetroxide in a 0.1 M cacodylate buffer, for 1 hour at room temperature. The specimens were then dehydrated through an increasing acetone series. The final series was 100% acetone, which was then infiltrated with 1:1 acetone: araldite CY212 resin overnight in a specimen rotator. The resin was then replaced by fresh resin twice, each change for a minimum of 3 hours. Once this procedure was complete, specimens were embedded in araldite CY212 resin and blocks were polymerised at 60^0C for 18 hours. Semi thin (1μm) sections were cut from the specimens using a Reichart-Jung Ultracut E ultramicrotome and floated onto distilled water and collected on glass slides. Ultra-thin sections (80-100nm) were cut using a Diatome diamond knife, floated onto distilled water, collected on copper grids and stained with 2% uranyl acetate and lead citrate for 10 minutes in each solution. The stained sections were viewed on a Phillips CM12 electron microscope. (All the reagents used in the TEM procedure were from Agar Scientific Ltd., Essex, England).

Results

Primary human MDCs were seeded at a density of 30million/5ml collagen gel, the gel was then plastic compressed and tethered to the CFM to measure the force generated over 24 hours. The resulting force contraction profile obtained is shown in Figure 64 and contrasted with the force contraction profile obtained from a normal (hyperhydrated) collagen gel with cells seeded at the same initial density.

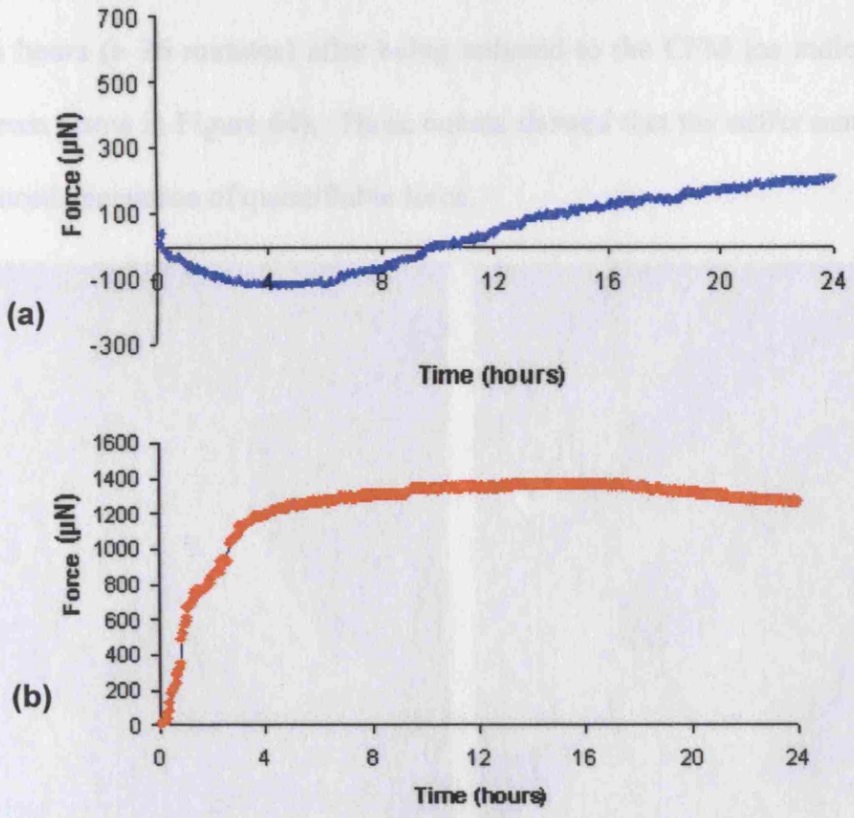


Figure 64: The force contraction profile obtained when 30 million MDCs were seeded in (a) plastic compressed (PC) and (b) normal 'hyper-hydrated' collagen gels. The initial negative force generation of the PC gel is due to stress-relaxation of the collagen gel after the process of plastic compression. The green arrow indicates onset of force generation. The force-time profiles are an average of 3 separate experiments.

The results show that the peak force generated as MDCs remodelled the PC collagen gel was 206μN (±20μN) this was significantly ($p<0.05$) lower than the peak force generated (1372μN) when the same number of cells remodelled the Normal (hyper-hydrated) collagen gel. The initial negative force generation is due to the stress-relaxation of the construct. Stress-relaxation was defined by Cheema (2004) and Karamichos (2006) as the unloading or continued deformation of the material (collagen gel) after loading. Initiation of contraction by the cells within the PC matrix began 6 hours (± 36 minutes) after being tethered to the CFM (as indicated by the small green arrow in Figure 64). These results showed that the stiffer matrix resulted in a reduced generation of quantifiable force.

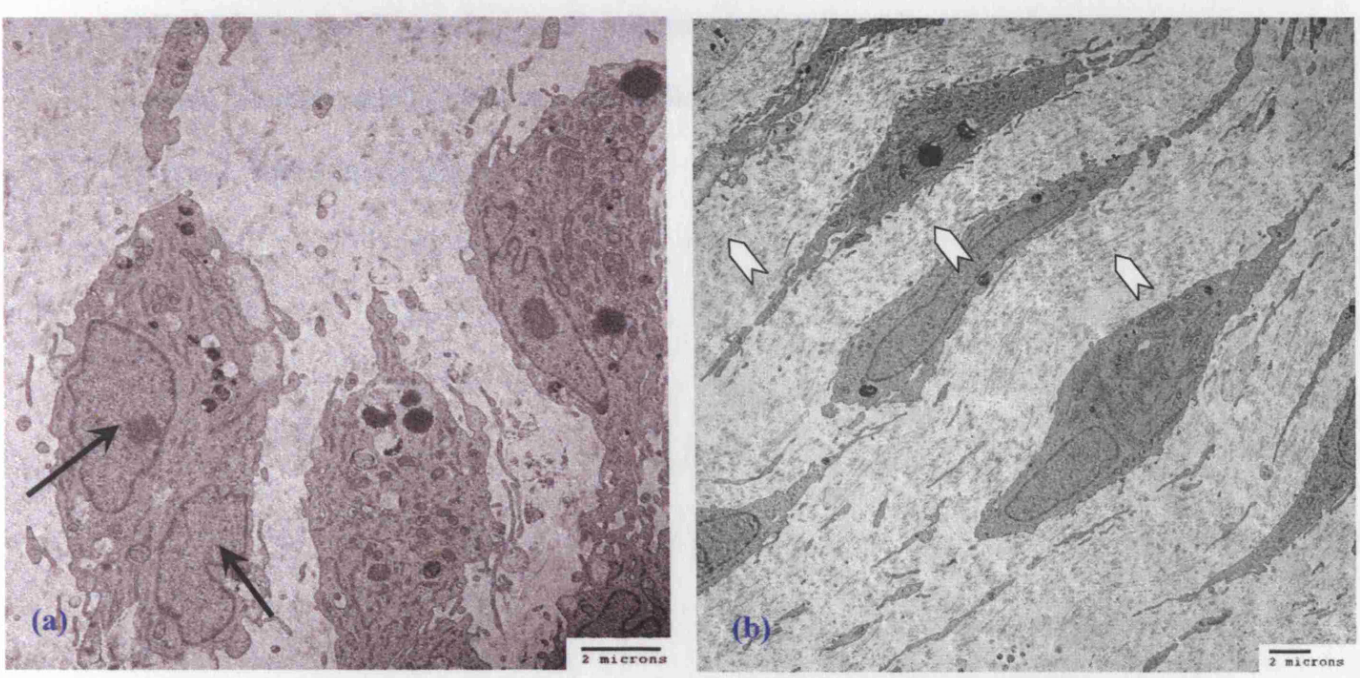


Figure 65: TEM images showing the effect of plastic compression on cell shape and collagen fibril density. MDCs were seeded at an initial density of 30 million/5ml collagen construct and images were taken after the gels were tethered to the CFM for 24 hours: (a) depicts cells seeded in the normal (hyper-hydrated) collagen gel and (b) the PC collagen gel. The embedded cells in the

normal gel are larger and contain more than one nuclei (shown by black arrows). However, the cells in the PC gel are smaller, with less cytoplasm. In the plastic compressed constructs there is an increased number of collagen fibrils clearly identifiable by the white arrow heads.

The next part of this study was to investigate and qualitatively analyse the effect of the process of plastic compression on cell shape and collagen fibril density. From the TEM images in Figure 65 (b) it can be clearly seen that the process of eliminating liquid from the hyper-hydrated collagen gel resulted in an increase in both cellular and collagen fibril density. Further, cell shape is affected by the process of PC, the cells in the normal 'hyper-hydrated' collagen constructs are larger, and in some cases, contain multiple nuclei, whereas the cells in the PC constructs are smaller, more compact, with less cytoplasm.

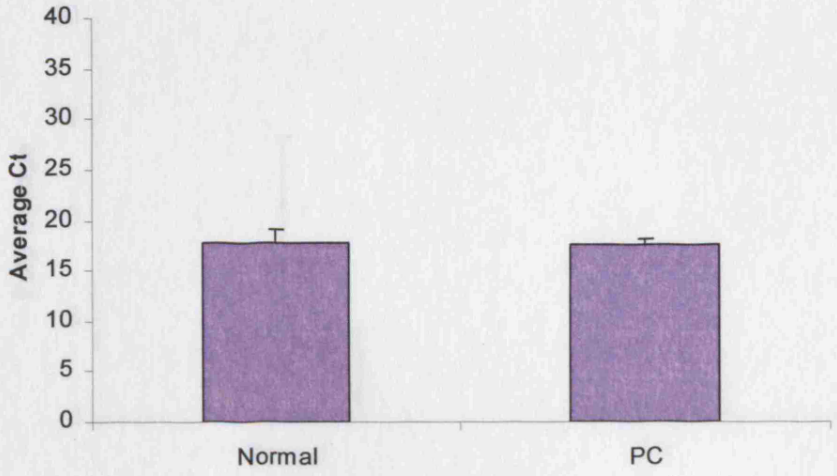


Figure 66: The average Ct of the internal control gene. GAPDH was validated to ensure that the experimental treatment did not effect its expression. Standard deviation was used for error bars (n=6+). There was no significant difference in gene expression so GAPDH was used as the internal control in this study.

The final aspect of this study was the molecular output. The hypothesis was that the process of plastic compression would promote the expression of muscle specific genes by increasing the incidence of cell-cell contact. Real time quantitative polymer chain reaction was used to test this hypothesis using the gene expression of myogenin and the myosin heavy chain isoforms (MYHC-IIX/D and MYHC-β) as markers of myogenic cell differentiation. As discussed earlier, the internal control gene was properly validated for each experiment to determine that gene expression was unaffected by the experimental treatment (Livak and Schmittgen, 2001). No significant difference ($p=0.3505$) was found in GAPDH expression between normal and PC collagen constructs (Figure 66). Therefore GAPDH was verified as an appropriate housekeeping gene in this study.

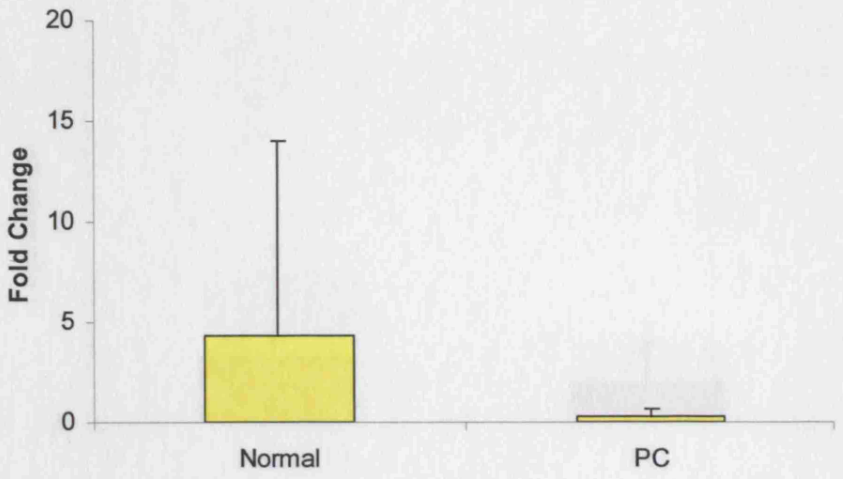


Figure 67: Myogenin gene expression is shown for MDCs seeded at 30 million/5ml collagen gel (n=6+) in Normal and PC constructs. Standard deviation is used for error bars.

The results in Figure 67 suggest that PC causes a down-regulation of myogenin after 24 hours in culture. However, these results were not statistically significant ($p=0.3379$) and no solid conclusions can be drawn from the data presented. Figures 68 (a) and (b) show that the gene expression of MYHC-IIX/D ($p=0.6286$) and MYHC-β ($p=0.3837$) were not significantly different in normal or plastic compressed constructs. These results suggest that the process of plastic compression does not promote the gene expression of markers of myogenic differentiation and a larger number of samples should be investigated. This could be correlated with the compacted cell shape which may hamper cell-cell communication within the stiffer matrix and thus reduce expression of the gene milieu that encodes for myogenic cell differentiation.

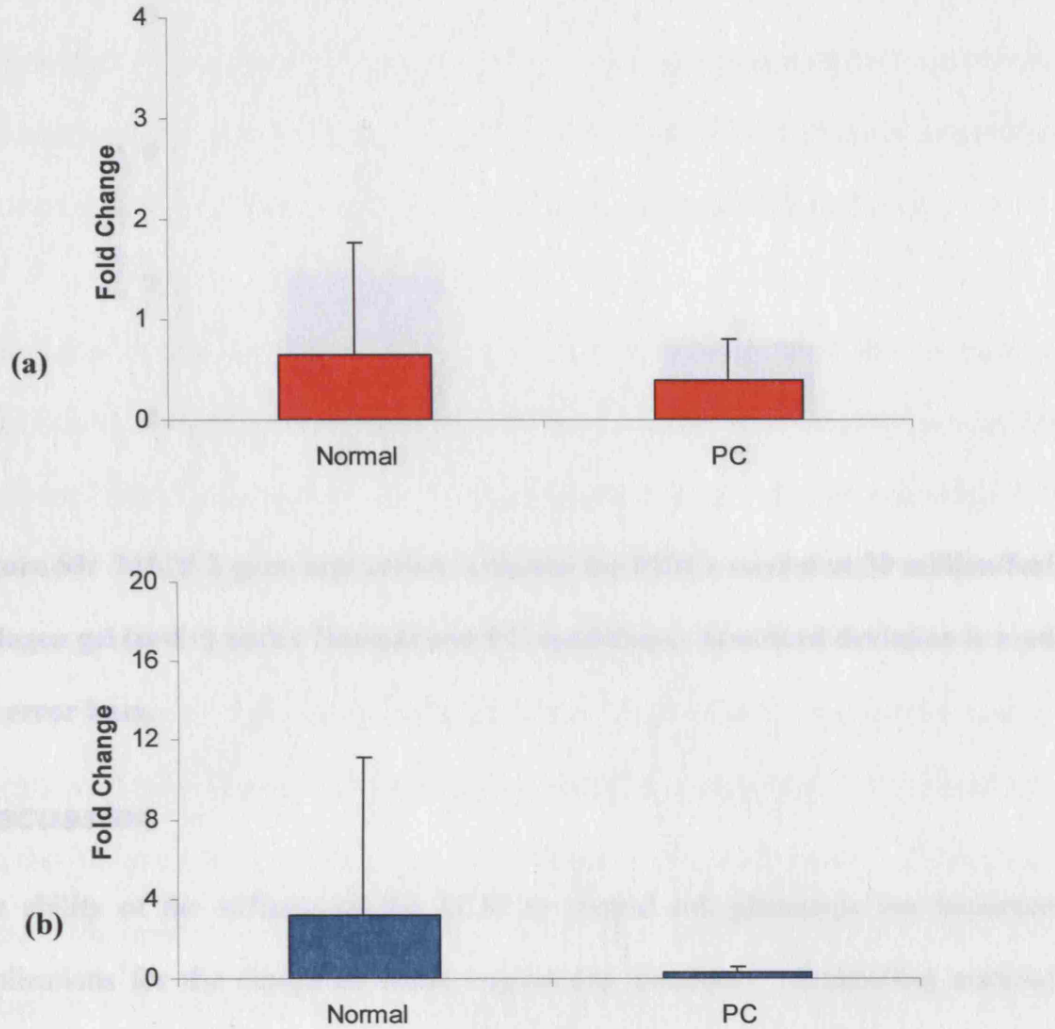


Figure 68: The gene expression of myosin heavy chain isoforms (a) MYHC-IIX/D (fast; red) and (b) MYHC-β (slow; green) for normal and plastic compressed MDC seeded constructs. Standard deviation is used for error bars (n=6+ each).

The final aspect of this study was to test the hypothesis that the gene expression of the matrix degrading enzyme MMP-2 would be up-regulated in response to the stiffer plastic compressed matrix. The results in Figure 69 show that the gene expression of MMP-2 in Normal and PC MDC seeded collagen gels were not significantly different ($p=0.2668$).

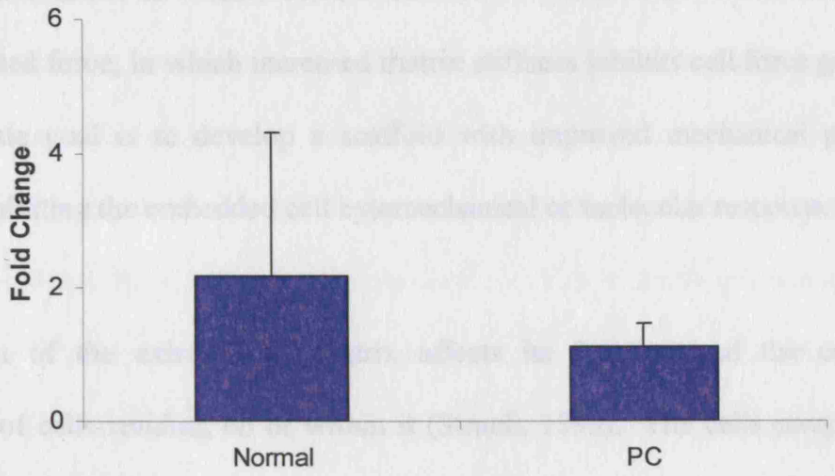


Figure 69: MMP-2 gene expression is shown for MDCs seeded at 30 million/5ml collagen gel (n=6+) under Normal and PC conditions. Standard deviation is used for error bars.

Discussion

The ability of the stiffness of the ECM to control cell phenotype has important implications for the design of tissue engineering matrices. Controlling material stiffness may allow one to regulate several cell responses, in essence tailoring the material properties to elicit the desired cell response. In this study the novel process of plastic compression was used to rapidly improve the material strength of the scaffold for skeletal muscle tissue engineering applications. The TEM images showed increased collagen fibril density of the stiffer matrix, cell shape was also affected by PC resulting in compact cells with smaller nuclei and cytoplasm. The response of the cells to this new, stiffer matrix resulted in significantly reduced measurable force and a delayed onset of force generation. Karamichos *et al.* (2007) similarly showed that increasing the stiffness of collagen type I lattices by uniaxial loading resulted in higher fibril orientation within it which resulted in significantly lower measurable contraction forces generated by the embedded cells (human dermal

fibroblasts). There is an evident correlation between matrix stiffness and endogenous cell generated force, in which increased matrix stiffness inhibits cell force generation. The ultimate goal is to develop a scaffold with improved mechanical properties, without inhibiting the embedded cell cytomechanical or molecular response.

Modulation of the extracellular matrix affects its function and the consequent behaviour of cells residing on or within it (Strueli, 1999). The cells composing the engineered tissue must express the appropriate genes to maintain the tissue specific function of the engineered tissue (Rosso *et al.*, 2004), for example, MDCs within an engineered muscle tissue construct must be induced to express the genes encoding terminal myogenic cell differentiation. The stiffer plastic compressed matrix implemented appeared to inhibit muscle specific gene expression. The hypothesis was that the process of plastic compression would both increase matrix stiffness and increase the incidence of cell-cell contact within the matrix, subsequently increasing myogenic cell differentiation and expression of myogenin. However, the results showed no significant difference in the gene expression of myogenin, or the myosin heavy chain isoforms, suggesting that the improved quality of matrix stiffness actually impedes development of myotubes, and subsequent myofiber types, in the 3D construct.

ECM remodeling through its synthesis, restructuring as well as degradation, is the key to understanding how different cell types maintain tissue integrity *in vivo* (Strueli, 1999). MMP-2 is the matrix degrading enzyme that has been monitored throughout this thesis as a marker of extracellular matrix remodeling. This study has shown that when primary human MDCs were seeded in stiffer matrices the levels of MMP-2

mRNA were not significantly altered. Karamichos *et al.* (2007) recently used mechanical loading to increase the stiffness of collagen constructs (seeded with human dermal fibroblasts) and showed that gene expression of MMP-2 was significantly up-regulated in stiffer constructs. In contrast, the results presented in this study indicate that increased stiffness does not elicit a matrix remodeling cellular response. Clearly, MMP-2 mRNA expression, in response to increased matrix stiffness, is dependent on cell type (e.g. MDCs) and experimental methodology (e.g. PC). In terms of experimental methodology, Karamichos *et al.* (2007) used mechanical pre-loading to increase matrix stiffness, these mechanical cues may have been responsible for the up-regulation of MMP-2 mRNA rather than the ensuing matrix stiffness per se. This study highlights the importance of investigating the molecular response of different cell types to the increased stiffness of the scaffolds used in tissue engineering applications.

Conclusions

- This study utilised the process of plastic compression to create a stiffer matrix for tissue engineering applications. The forces generated by MDCs seeded within this matrix were significantly reduced, compared to the forces generated when MDCs were seeded in normal 'hyper-hydrated' collagen matrices. That is, a stiffer matrix resulted in a reduced generation of quantifiable force.

- Using TEM this study qualitatively showed that the process of plastic compression caused an increase in collagen fibril density and the embedded cells were more compact, smaller, with visibly less cytoplasm, in PC gels.

- The process of plastic compression did not promote the expression of markers of myogenic terminal differentiation (myogenin, MYHC-IIX/D and MYHC-β).

- The mechanosensitive gene MMP-2, an established marker of matrix remodeling, was not upregulated in stiffer plastic compressed constructs.

General Discussion

Tissue engineering skeletal muscle involves inducing the fusion of myoblasts to myotubes *in vitro*, recapitulating the *in vivo* conditions during myogenesis. The tissue engineer attempts to guide the cellular organization and development in 3D tissue culture by providing the myogenic cells with the appropriate environmental cues (Kosnik *et al.*, 2003). However, to date, a functional tissue substitute for skeletal muscle has not yet been developed and to achieve this goal it is necessary to investigate new approaches for culturing differentiated skeletal muscle tissue *in vitro* (Bach *et al.*, 2004). To successfully tissue engineer skeletal muscle a number of parameters must be considered including cell source, scaffold, gene expression, biological cues (cell-cell interactions) and mechanical cues (cell-matrix interactions). In this study primary human craniofacial muscle derived cells were seeded in a 3D collagen type I scaffold to tissue engineer a craniofacial muscle construct and investigated the effect of biological and mechanical cues on cellular cytomechanical (force generation) and molecular (gene expression) response.

1. Cell Source

The source of the cells used for skeletal muscle tissue engineering depends on the intended use of the tissue engineered construct. Myogenic cells lines, including the C2C12 cell line from mouse muscle sarcoma (Okano and Matsuda, 1998; van Wachem *et al.*, 1996; Vandenburgh *et al.*, 1998, Cheema *et al.*, 2003) and the murine G8 cell line (Mulder *et al.*, 1998), have been used for their cost effectiveness, ease of use and uniformity. However, these cells have disadvantages, primarily, they are essentially immortalised cells and it is not possible to use such cells in clinical

applications. In the absence of immune suppression, non-autologous cells will be identified by the host and rejected within several weeks (Kosnik *et al.*, 2003). Another limitation of using cell lines is that the cells do not exhibit all of the same behaviours as primary myogenic cells *in vitro* (Irnitchev *et al.*, 1998). For example, Dennis *et* al., (2001) showed that muscle constructs engineered from myogenic cell lines exhibited significantly lower contractility and prolonged relaxation times after cessation of electrical stimulation compared with constructs engineered from primary adult rodent cells. The use of myogenic cell lines for muscle tissue engineering studies must be complemented with primary cell studies.

Autologous cells are required for clinical applications in which engineered skeletal muscle can be implanted into the host without a barrier to immune response or the need for immune suppression (Kosnik *et al.*, 2003). Consequently, three dimensional skeletal muscle constructs have been engineered from primary cultures of avian (Strohman *et al.*, 1990; Swasdison and Mayne, 1992; Vandenburgh *et al.*, 1991), rodent (Dennis and Kosnik, 2000; Powell *et al.*, 1999; Vandenburgh *et al.*, 1996, 1999) and human (Powell *et al.*, 1999) myogenic cells. A unique aspect of this study, considering the potential clinical application of the tissue engineered craniofacial muscle construct, was the use of primary human masseter muscle derived cells (MDC).

Critical choices on the source of cells for the ideal tissue engineered muscle construct should have a combination of fibroblasts, myoblasts and satellite cells (Lewis *et al.*, 2008). Immunostaining for desmin showed that the MDC cultures derived from the human masseter were a co-culture of myogenic and non-myogenic cells. However,

primary myoblasts cell cultures need to be expanded to a great extent in order to obtain large volumes of tissue engineered skeletal muscle (Bach *et al.*, 2004). Further, the processes of isolation, purification and proliferation of primary myoblast are labour intensive and costly (Kosnik *et al.*, 2003). This was one of the major limitations of this study. The process of culturing and isolating the required volume of primary human myogenic cells for tissue engineering craniofacial muscle constructs was labour intensive and expensive, future research should scale down the muscle model an order of magnitude (i.e. from mls to µls and from millions of cells to hundred thousands of cells).

Only one other group has attempted to use primary human muscle cells to tissue engineer skeletal muscle. Powell *et al.* (1999; 2002) seeded MDCs from the vastus lateralis muscle into a matrigel:Collagen gel mixture. However, matrigel™ is an extract from the Engelbreth-Holm-Swarm mouse sarcoma and contains extracellular matrix proteins and growth factors in undefined concentrations. Further, it has the ability to change gene expression in cells (Bach *et al*, 2004). Due to its origin its utility, matrigel™ is not appropriate for a clinical setting and thus limited to experimental models. A distinctive aspect of the model presented in this study was the choice of scaffold material.

2. *Scaffolds*

Scaffolds are artificial extracellular matrixes (ECMs) that define the initial geometry, structure and mechanical properties of the engineered skeletal muscle construct, they also have an essential role in the control and maintenance of cellular function,

including proliferation and fusion (Kosnik *et al.*, 2003; Lewis *et al.*, 2008). Ideally, matrices should provide a high surface area for cell-matrix interactions, sufficient space for extracellular matrix (ECM) regeneration and minimal diffusion constraints during *in vitro* culture (Rebel *et al.*, 1994; Ye *et al.*, 2000a). Moreover the matrix should be resorbable once it has served its purpose of providing a basic structure for the developing tissue (Freed *et al.*, 1994; Mikos *et al.*, 1994).

Myogenic cells exhibit anchorage dependent growth (Stoker *et al.*, 1968) and do not proliferate in the absence of a suitable substrate on which to adhere. They can be cultured on substrates of plastic, glass, or flexible membranes that have been coated with collagen, fibronectin, laminin, matrigel^{TM}, or combinations of these molecules (Chiquet *et al.*, 1979; Turner, 1986). Scaffolds used in skeletal muscle tissue engineering include collagen gels (Chromiak *et al.*, 1998; Okano and Matsuda, 1997; Vandenburgh *et al.*, 1999), collagen-matrigel gels (Powell *et al.*, 1999; 2002), alginate hydrogels (Rowley *et al.*, 1999), polyurethanes (Mulder *et al.*, 1998) and phosphate-based glass fibers (Shah *et al.*, 2005)

In this study type I collagen was the scaffold of choice, it is the most abundant and best studied collagen, and it is also highly conserved across species. In most organs and notably in tendons and fascia, type I collagen provides tensile stiffness and in bone, it defines considerable biomechanical properties concerning load bearing, tensile strength, and torsional stiffness (Gelse *et al.*, 2003). In addition to their key functional role as biomechanical structures, the collagens interact directly with cells to influence adhesion, growth, differentiation and many other cellular activities (Brown and Phillips, 2007). Collagen provided the ideal starting point as a scaffold to tissue

engineer a new muscle construct and this study showed that primary human MDCs could be embedded in the 3D matrix with cell viability being maintained (Chapter 2). Further, we showed that increased matrix stiffness resulted in a reduction in endogenous cell generated force and no significant change in gene expression of markers of myogenic cell differentiation and matrix remodeling (Chapter 5). Future investigations to improve the material strength of scaffolds used in tissue engineering should investigate the correlation between matrix stiffness and cellular response, testing a range of matrix stiffness to cellular response to establish the threshold at which matrix stiffness begins to inhibit cellular cytomechanical response.

3. Gene expression

Skeletal myogenesis is the process by which myoblasts proliferate, organize in three dimensions, and differentiate to form skeletal muscle tissue de novo (Kosnik *et al.*, 2003). Many of the steps involved in the differentiation of myoblasts to their subsequent fusion into multinucleate muscle fibers can be studied in molecular terms, with expression of specific transcription factors controlling each developmental event (Bach *et al.*, 2003). Molecular biology is guided by the desire to understand how cells and tissues develop their unique organic qualities, including the ability to change shape, move and grow (Ingber, 1998). Skeletal myoblasts express their genes in the context of their environment and developmental history (Kosnik *et al.*, 2003). In the first part of this study (Chapter 1) it was shown that in 2D cultures containing differentiated myogenic cells expressed myogenin (the established marker of myogenic cell differentiation), whereas, cultures containing single cells did not express the gene. Therefore, myogenin was used in subsequent 3D studies as a

quantitative marker of differentiation. In the chapters that followed the effect of specific environmental cues were tested, including biological cues (i.e. cell separation, increased cell density and extended time in culture) and mechanical cues (external mechanical loading and plastic compression), on expression of this gene in a 3D matrix, further, additional genes MYHC-β, MyHC-IIX and MMP-2 were evaluated to monitor fiber type determination and matrix degradation. The focus of this thesis was to quantitate the cytomechanical and molecular response of MDCs embedded in collagen type I constructs, however, qualitative analysis was limited due to time and resources. Future investigations could utilize qualitative measures, such as confocal microscopy, to visually identify multinucleated cells within the 3D constructs.

The tissue engineer uses environmental cues to direct the three-dimensional organization, proliferation, and differentiation of the cells that will form the engineered skeletal muscle. The appropriate environment cues lead to changes in gene expression within the cell, which results in changes in cellular metabolism, growth, and organization (Figure 70). The key to controlling the three dimensional organization, proliferation, and differentiation of the cells is to provide the appropriate cues that will result in the desired outcome for the tissue (Kosnik *et al.*, 2003).

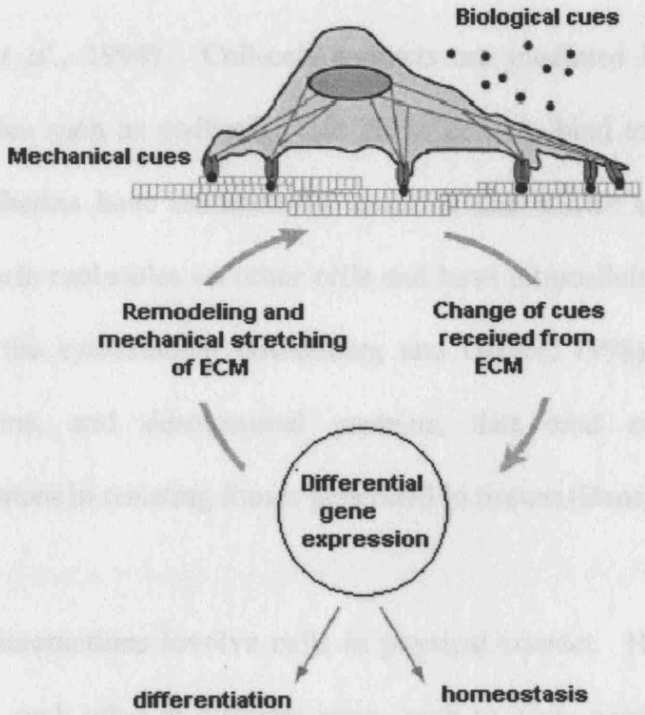


Figure 70: Cells are guided by mechanical and biological cues received from their environment. Upon cell adhesion to 3-D matrices, a spatial redistribution and clustering of transmembrane receptors occurs, which in turn triggers the restructuring of the cytoskeleton. Cell signaling pathways can be triggered that regulate gene expression and ultimately cell migration and differentiation. Cytoskeletal reconfiguration leads to the remodeling of the ECM. Cells are then able to physically remodel the ECM, which in turn changes the set of cues cells receive from their environment (Adapted from Vogel and Baneyx, 2003).

4. *Biological cues (cell-cell interactions)*

The fusion of myoblasts is central to the successful differentiation of skeletal muscle (Lewis *et al.*, 2001; Cheema, 2004). During fusion *in vitro*, myoblasts undergo partial detachment from their plastic substratum forming long linear arrays and it has been suggested that cell-cell interactions are more important than cell-matrix interactions at

this stage (Gu *et al.*, 1994). Cell-cell contacts are mediated by transmembrane adhesion molecules such as cadherins that allow cells to bind to and interact with other cells. Cadherins have extracellular domains that adhere to the extracellular domains of cadherin molecules on other cells and have intracellular domains that are continuous with the cytoskeleton (Goichberg and Geiger, 1998). Proteins such as cadherins, catenins, and desmosomal proteins, that bind cells together, are intrinsically important in resisting forces generated in tissues (Banes *et al.*, 2003).

Muscle cell-cell interactions involve cells in physical contact. However, cells may also interact with each other in different ways, such as when neighbouring cells (i.e. fibroblasts/non-myogenic cells) secrete mitogens, growth factors or ECM molecules that affect other cells (Kosnik *et al.*, 2003). Dennis *et al.* (2001) defined *in vitro* skeletal muscle tissue engineering as culturing isolated myoblasts and fibroblasts in an environment conducive to the formation of a three-dimensional tissue construct (myooid) (Dennis *et al.*, 2001), highlighting the critical role that fibroblasts have in assisting in extracellular matrix formation during *in vitro* myogenesis (Mayne *et al.*, 1989; Shansky *et* al., 1997; Dennis *et al.*, 2001). Their results indicated that C2C12 myoblasts did not form myooids when monocultured, yet they formed myooids with some success when cocultured with fibroblasts, substantiating the critical role of fibroblasts in the formation of myooids (Dennis *et al.*, 2001).

Throughout this thesis biological cues have been investigated in the development of a tissue engineered craniofacial muscle construct. Firstly, cell-cell interactions between myogenic and non-myogenic MDCs were studied. Only one study has previously investigated the role of primary human myogenic and non-myogenic cells in 2D

myogenesis (Sinanan *et al*, 2004) and no studies have been reported on the separation and culture of these cells in a 3D-matrix. The results of this study (Chapter 2) have shown that the presence of non-myogenic cells in 3D muscle tissue engineering enhances force generation and gene expression of muscle specific genes. The significant impact of this work is that it has added to current understanding of the interplay between myogenic and non-myogenic cells for successful *in vitro* myogenesis. Indicating that future attempts to tissue engineer skeletal muscle should utilize the heterogenous mixture of primary human MDCs so that the appropriate biological cues are maintained.

The next aspect of cell-cell interaction investigated in this thesis was the effect of increasing the incidence of cell-cell contact of myogenic cells by seeding the cells at high densities. An important finding of this study (Chapter 4) was the significant up-regulation of the MyHC-β gene expression with increasing cell density indicative of a 'community effect' influencing the composition of the gene milieu that determines the resulting muscle fiber type.

The final biological cue investigated was the effect of extending the period of time that the cells were in culture to encourage maturation of the constructs through cell-cell interactions. The results showed an apparent up-regulation of all muscle specific gene expressions (i.e. myogenin, MyHC-IIX and MyHC-β). Indicating that future attempts to tissue engineer skeletal muscle should employ longer periods (> 6 days) in 3D culture.

5. Mechanical cues (cell-matrix interactions)

Tissues depend on mechanical cues for homeostasis and matrix remodeling (Banes *et al.*, 1995; 2001; Frost, 1988). Mechanical forces generated in the growing embryo and in the active adult play a crucial role in organogenesis as complex patterns of mechanical loading are applied to skeletal myoblasts and myofibres through the elongating skeleton and by foetal movements (Lewis *et al.*, 2008). Vandenburgh *et al.* (1987) showed that repetitive loading stimulated muscle cell hypertrophy, whereas no loading results in tissue atrophy. Vandenburgh *et al.*, (1991) went on to show that in tissue engineered muscle constructs mechanical stimulation encouraged cell proliferation, myotube orientation and myotube longitudinal growth. Another group has shown that mechanical stimulation of 3D collagen constructs of fused C2C12 myoblasts resulted in up-regulation of IGF-I gene splicing and production, and it is likely that changes in myofibre size may be affected through this mechanism (Cheema *et al.*, 2004). However, the mechanisms by which mechanical cues lead to eventual biochemical and molecular responses remain undefined, and further studies will undoubtedly provide new insight into constructing tissues for artificial organs (Huang *et al.*, 2004).

An important element in the design of functional engineered skeletal muscle is an understanding of the force transmission from muscle cells to the surrounding matrix (Faulkner and Dennis, 2003). The process of converting physical forces into biochemical signals and integrating these signals into the cellular responses is referred to as mechanotransduction (Figure 71; Huang *et al.*, 2004).

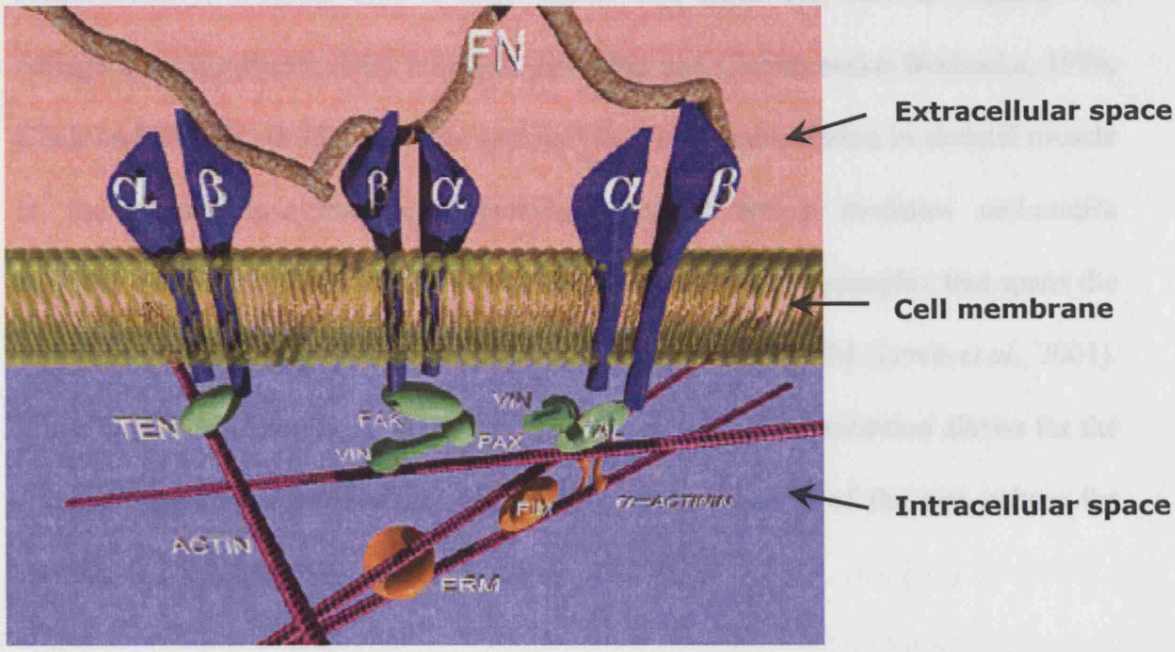


Figure 71: Mechanotransduction is the process of converting extracellular mechanical signals into an intracellular response. Transmembrane proteins include mechanosensitive ion channels (green) and signaling molecules such as integrins (blue). Clusters of integrins form focal adhesions, which play a vital role in mechanotransduction. (Key: FN, fibronectin; α and β, α- and β-integrin dimer subunits; TEN, tensin; FAK, focal adhesion kinase; VIN, vinculin; PAX, paxillin; FIM, fimbrin; TAL, talin; ERM, ezrin-radixin-moesin proteins). Image is not drawn to scale (Source: Huang *et al.*, 2004).

Cell-cell and cell-matrix interactions are transmitted to the internal cellular structures, such as the cytoskeletal components (Huang *et al.*, 2004). One common pathway for force transmission is via the focal adhesions (clusters of integrins). Integrins, provide a direct mechanical linkage between the matrix and cell interior, they constitute a primary pathway for intracellular force transmission and therefore have been viewed as likely candidates for the initiating mechanosensing event (Huang *et al.*, 2004).

Extracellularly, integrins bind to short amino acid sequences, such as sequences in collagens, to the cytoskeletal network (Burridge and Chrzanowska-Wodnicka, 1996, Clark and Brugge, 1995). Another pathway for force transmission in skeletal muscle is the dystrophin-dystroglycan complex (DGC) which mediates cell-matrix interactions (Figure 72). The DGC is a multi-subunit protein complex that spans the sarcolemma connecting the cytoskeleton to the surrounding ECM (Lewis *et al.*, 2001). Since the cytoskeleton is in contact with the nucleus, this organization allows for the transduction of extracellular signals directly to the nucleus of the cell, where the signal can effect gene expression (Wang *et al.*, 1993).

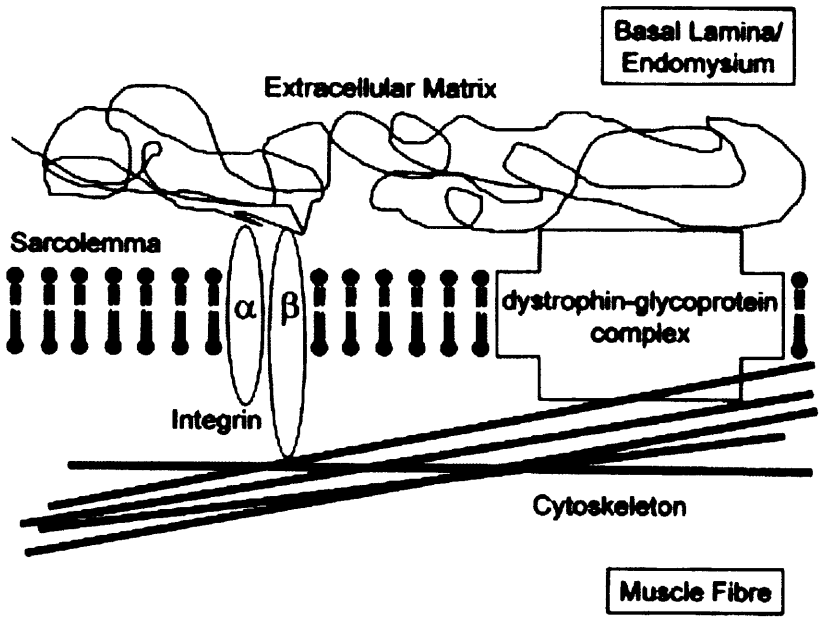


Figure 72: Cell-matrix interactions between skeletal muscle and its surrounding connective tissue are mediated via integrins and the dystrophin-dystroglycan complex (Source: Lewis *et al.*, 2001).

Further, mechanotransduction can be regulated outside the cell by extracellular matrix proteins and proteolytic enzymes, more specifically, proteolytic degradation (by MMP-2) of the ECM is an essential feature of repair and subsequent remodelling

stages (Karamichos, 2006). MMPs are known for their role in wound healing, angiogenesis, embryogenesis, and in many pathological process such as tumour metastasis (Stetler-Stevenson, 2001). Clearly therefore, protease production is essential in ECM remodeling and understanding of the process and the specific proteases involve in mechano-responsive changes will be critical in *in vitro* and/or *in vivo* models (Karamichos, 2006).

The primary mechanical cue investigated in this thesis was the effect of external mechanical loading (Chapter 3) on muscle specific (myogenin, MyHC-β and MyHC-IIX/D) and MMP-2 gene expression in the MDC seeded 3D construct; the results indicated no significant change due to the applied mechanical cues. On the cellular level, responses to mechanical loads are now well documented and the challenge is to apply the appropriate strain (there will be an optimum magnitude, frequency, duration and type of load) to cells and measure tissue-specific outcomes (Banes *et al.*, 2003). Future studies are required and one must test a number of responses and gene read-outs before a general statement can be made with certainty. Understanding the hierarchy of load signals required to achieve a positive outcome is also an important consideration.

The matrix-integrin-cytoskeleton linkage also exerts force from the inside outward, onto the matrix (Banes *et al.*, 2003). Cells can apply measurable mechanical forces to substrates resulting in contraction of the matrix. Throughout this study we quantified these forces and characterised for the first time the force contraction profiles of primary human MDCs seeded in 3D constructs at different densities, establishing the optimal cell seeding density (30 million/5ml collagen construct) for maximum force

generation. The secondary mechanical cue investigated in this thesis was the effect of increasing matrix stiffness by application of the novel process of PC (Chapter 5). The results indicated that the cells in PC constructs generated less measurable force and there was no significant difference, though an apparent down-regulation, in all gene expressions measured, concluding that the stiffer matrix did not promote myogenic cell differentiation, nor matrix remodelling.

Conclusions and Future Research

From all the above studies it can be concluded that cell-cell signals (biological cues) are more important than cell-matrix signals (mechanical cues) in 3D myogenic cell differentiation. The important aspect of this study is that it extends our limited knowledge of the parameters that define successful craniofacial skeletal muscle tissue engineering. Progress has been made in the construction of muscle organoids by application of mechanical and biological signals to ensure correct alignment and fusion, however, a fully functional skeletal muscle needs to be able to interact and respond to its environment (Lewis *et al.*, 2008). The primary human neo-muscle model presented in this study was extremely basic. There was no neural input, and no effects of systemic regulation. However, the advantage it offers over the *in vivo* situation is that it is possible to alter one parameter at a time and therefore it is possible to begin to understand the mechano-transduction mechanisms of muscle derived cells. By understanding this basic model, advances can be made in the future.

Skeletal muscle tissue engineering has become a general model for understanding many fundamental principals of development, including mechanisms for cell

differentiation, morphogenesis and the antagonism between growth and differentiation (Bach *et al.*, 2004). Further, successes to date have indicated that the ideal solution will probably entail a combination of approaches that recognize the system requires directional cues generated by non-permanent stimuli (e.g. scaffolds), mechanical pre-conditioning, the correct combinations of cells (e.g. myogenic, connective tissue, vascular), attachments to nerve and bone (via tendons) and a neural input (Lewis *et al.*, 2008).

While the goal of using *in vitro* engineered skeletal muscle for functional work *in vivo* is still years away form clinical use, near term applications of the rapidly evolving tissue engineered skeletal muscle technology are possible (Kosnik *et al.*, 2003). These include their use as implantable protein delivery devices (Vandenburgh *et al.*, 1996), high content drug screening for skeletal muscle wasting disorders Vandenburgh *et al.*, 1999) and as mechanical *ex vivo* actuators, and for pharmaco-genomic screening for testing patient's reactions to drugs.

In conclusion, the most important area for future research on functional tissue engineering of skeletal muscle is in the provision of optimum environmental cues to ensure optimum development of muscle constructs (Faulkner and Dennis, 2003). Requirements are for a better understanding of the control of the gene expressions of the transcriptional factors and proteins that control the specification and differentiation of muscle cells (Sabourin and Rudnicki, 2000) in 3D cultures. This investigation of mechanical and biological cues, and the resulting gene expression, presented in the current thesis has added significant knowledge to the field of tissue engineering a functional skeletal muscle construct.

References

Abatangelo G, Brun P, Radice M, Cortivo R, Auth MKH. 2001. 'Tissue engineering.' In: Barbucci R Ed. *Integrated biomaterials science* New York: Kluwer Academic pp 885–945

Abou Neel EA, Cheema U, Knowles JC, Brown RA, Nazhat SN. 2006. 'Use of multiple unconfined compression for control of collagen gel scaffold density and mechanical properties.' *Soft Matter* 2: 986-992

Acarturk TO, Peel MM, Petrosko P, LaFramboise W, Johnson PC, DiMilla PA. 1999. 'Control of attachment, morphology, and proliferation of skeletal myoblasts on silanized glass.' *J Biomed Mater Res* 44: 355-370

Adams JC and Watt FM. 1993. 'Regulation of development and differentiation by extracellular matrix.' *Development* 117: 1183-1198

Agbulut O, Noirez P, Beauomont F and Butler-Browne G. 2003. 'Myosin heavy chain isoforms in postnatal muscle development of mice.' *Biology of the Cell* 95:399-406

Alberts B, Bray D, Lewis J, Raff M, Roberts K and Watson JD. 1998. 'Molecular biology of the cell.' *Third Edition, Garland Publishing, Inc.* New York and London

Alhadlaq A and Mao JJ. 2003. 'Tissue-engineered neogenesis of human-shaped mandibular condyle from rat mesenchymal stem cells.' *J Dent Res* 82:951–956

Alhadlaq A, Elisseeff JH, Hong L, Williams CG, Caplan AI, Sharma B, Kopher RA, Tomkoria S, Lennon DP, Lopez A and Mao JJ. 2004. 'Adult stem cell driven genesis of human-shaped articular condyle.' *Ann Biomed Eng* 32:911–923

Alhadlaq A and Mao JJ. 2005. 'Tissue engineered osteochondral constructs in the shape of an articular condyle.' *J Bone Joint Surg Am* 87:936–944

Allen DL, Harrison BC and Leinwand. 2000. 'Inactivation of myosin heavy chain genes in the mouse: diverse and unexpected phenotypes.' *Microsc Res Tech* 50:492-499

Allen DL and Leinwand LA. 2001. 'Postnatal Myosin heavy chain isoform expression in normal mice and mice null for IIb or IId myosin heavy chains.' *Developmental Biology* 229: 383–395

Alsberg E, Hill EE and Mooney DJ. 2001. 'Craniofacial tissue engineering.' *Crit Rev Oral Biol Med* 121.:64-75

Andrés V and Walsh K. 1996. 'Myogenin expression, cell cycle withdrawal, and phenotypic differentiation are temporally separable events that precede cell fusion upon myogenesis.' *J Cell Biol* 1324.:657-666

Arosarena O. 2005. 'Tissue engineering.' *Current Opinion in Otolaryngology & Head and Neck Surgery* 13:233—241

Asanuma K, Magid R, Johnson C, Nerem RM, Galis ZS. 2003. 'Uniaxial strain regulates matrix-degrading enzymes produced by human vascular smooth muscles.' *Am J Physiol Heart Circ Physiol* 284: H1774–H1778.

Atchely WR and Fitch WM. 1997. 'A natural classification of the basic helix-loop-helix class of transcription factors.' *Proc Natl Acad Sci* USA 94: 5172-6

Auluck A, Mudera V, Hunt NP, Lewis MP. 2005. 'A three-dimensional in vitro model system to study the adaptation of craniofacial skeletal muscle following mechanostimulation.' *Eur J Oral Sci* 113: 218–224

Bach AD, Beier JP and Stark GB. 2003a. 'Expression of Trisk 51, agrin and nicotinic-acetylcholine receptor ε-subunit during muscle development in a novel three-dimensional muscle neuronal co-culture system.' *Cell Tissue Res* 314:263-274

Bach AD, Stem-Straeter J, Beier JP, Bannasch H and Stark GB. 2003b. 'Engineering of muscle tissue.' *Clin Plast Surg* 30: 589-599

Bach AD, Beier JP, Stern-Staeter J and Horch RE. 2004. 'Skeletal muscle tissue engineering.' *J Cell Mol Med* 84.:413-422

Bach AD, Arkudas A, Tjiawi J, Polykandriotis E, Kneser U, Horch RE, Beier JP. 2006. 'A new approach to tissue engineering of vascularized skeletal muscle.' *J Cell Mol Med* 10 3.: 716–726

Bacou F, Rouanet P, Barjot C, Janmot C, Vigneron P and d'Albis A. 1996. 'Expression of myosin isoforms in denervated, cross-reinnervated, and electrically stimulated rabbit muscles.' *Eur J Biochem* 236:539-547

Bader DL and Lee DA. 2005. 'Mechanical conditioning of cell-seeded constructs for solft tissue repair- are optimisation strategies possible?' In: Chaudhuri JB and Al-Rubeai M. Eds *Bioreactors for Tissue Engineering* Springer Science, Netherlands Chapter 7; 165-192

Baj A, Bettaccini AA, Casalone R, Sala A, Cherubino P and Toniolo AQ. 2005. 'Culture of skeletal myoblasts from human donors aged over 40 years: dynamics of cell growth and expression of differentiation markers.' *Journal of Translational Medicine* 3:21-31

Baldwin KM and Haddad F. 2001. 'Effects of different activity and inactivity paradigms on myosin heavy chain gene expression in striated muscle.' *J Appl Physiol* 90:345-357

Banes A, Tsuzaki M, Yamamoto J, Fischer T, Brigman B, Brown T, Miller L. 1995. 'Mechanoreception at the cellular level: the detection, interpretation, and diversity of responses to mechanical signals.' *Biochem Cell Biol* 73: 349-365

Banes AJ, Weinhold P, Yang X, Tsuzaki M, Bynum D, Bottlang M, Brown T. 1999. 'Gap junctions regulate responses of tendon cells *ex vivo* to mechanical loading.' *Clin Orthop* 367: S356-s370

Banes AJ, Wall M, Garvin J, Archambault J. 2003. 'Cytomechanics: Signaling to mechanical load in connective tissue cells and its role in tissue engineering.' In: Guilak F, Butler DL, Goldstein SA, Mooney DJ Eds. Functional Tissue Engineering. Springer New York, p 318-334

Barany M. 1967. 'ATPase activity of myosin correlated with speed of muscle shortening.' *J Physiol* 213:455-474

Barroffio A, Aubry JP, Kaelin A, Krause RM, Hamann M and Bader CR. 1993. 'Purification of human muscle satellite cells by flow cytometry.' *Muscle Nerve* 16; 498-505

Beauchamp JR, Heslop L, Yu DSW, Tajbakhsh S, Kelly RG, Wernig A, Buckingham ME, Partridge TA and Zammit PS. 2000. 'Expression of CD34 and Myf5 defines the majority of quiescent adult skeletal muscle satellite cells.' *Journal of Cell Biology* 151:1221–1233

Beier JP, Stern-Straeter J, Foerster VT, Kneser U, Stark GB, and Bach AD. 2006. 'Tissue Engineering of Injectable Muscle: Three-Dimensional Myoblast-Fibrin Injection in the Syngeneic Rat Animal Model.' *Plastic & Reconstructive Surgery* 1185.:1113-1121

Bell E, Ivarsson B and Merrill C. 1979. 'Production of a tissue-like structure by contraction of collagen lattices by human fibroblasts of different proliferative potential *in vitro.' Proc Natl Acad Sci* 76:1274-1278

Belles-Isles M, Roy R, Dansereau G, Goulet M, Roy B, Bouchard JP and Tremblay JP. 1993. 'Rapid selection of donor myoblasts clones for muscular dystrophy therapy using cell surface expression of NCAM.' *Eur. J. Histochem* 37:375-380

Beningo KA, Dembo M and Wang Y. 2004. 'Responses of fibroblasts to anchorage of dorsal extracellular matrix receptors.' *Proc. Natl. Acad. Sci. U.S.A* 10152.:18024-18029

Bitar M, Salih V, Brown RA and Nazhat S. 2007. 'Effect of multiple unconfined compression on cellular dense collagen scaffolds for bone tissue engineering.' *J Mater Sci: Mater Med* 18:237–244

Blanton JR, Jr., Grant AL, McFarland DC, Robinson JP and Bidwell CA. 1999. 'Isolation of two populations of myoblasts from porcine skeletal muscle.' *Muscle Nerve* 22:43-50

Blau HM and Webster C. 1981. 'Isolation and characterization of human muscle cells.' *Proc. Natl. Acad. Sci. USA* 78: 5623-5627

Bolcato-Bellemin AL, Elkaim R, Abehsera A, Fausser JL, Haikel Y, Tenenbaum H. 2000. 'Expression of mRNAs encoding for alpha and beta integrin subunits, MMPs, and TIMPs in stretched human periodontal ligament and gingival fibroblasts.' *J Dent Res* 79: 1712–1716

Bonavaud S, Agbulut O, D'Honneur G, Nizard R, Mouly V and Butler-Brown G. 2002. 'Preparation of isolated human muscle fibers: a technical report.' *In Vitro Cell. Dev. Biol.—Animal* 38:66–72

Bottinelli R, Canepari M, Pellegrino MA, Reggiani C. 1996. 'Force velocity properties of human skeletal muscle fibres: myosin heavy chain isoform and temperature dependence.' *J Physiol* 495:573-586

Boudreault P, Tremblay JP, Pépin MF and Garnier A. 2001. 'Scale-up of a myoblast culture process.' *Journal of Biotechnology* 91:63-74

Boyd SB, Gonyea WJ, Finn RA, Woodard CE, and Bell WH. 1984. 'Histochemical Study of the Masseter Muscle in Patients with Vertical Maxillary Excess.' *J Oral Maxillofac. Surg.* 47:953-962

Bredman JJ, Wessels A, Weijs WA, Korfage JAM, Soffers CAS, and Moorman AFM. 1991. 'Demonstration of 'cardiac-specific.' myosin heavy chain in masticatory muscles of human and rabbit.' *Histochem J* 23: 160–70

Brown RA, Talas G, Porter RA, McGrouther DA and Eastwood M. 1996. 'Balanced mechanical forces and microtubule contribution to fibroblasts contraction.' *J Cell Physiol* 1693.:439-447

Brown RA, Prajapati R, McGrouther DA, Yanna IV and Eastwood M. 1998. 'Tensional Homeostasis in Dermal Fibroblasts: Mechanical Responses to Mechanical Loading in Three-Dimensional Substrates.' *J Cell Physiol.* 1753.:323-332

Brown RA, Wiseman M, Chuo C-B, Cheema U and Nazhat SN. 2005. 'Ultrarapid Engineering of Biomimetic Materials and Tissues: Fabrication of Nano- and Microstructures by Plastic Compression.' *Adv. Func. Mat.* 15:1762-1770

Brown RA. 2006. 'Cytomechanics in Connective Tissue Repair and Engineering.' In: Chaponnier C, Desmoulière A, Gabbiani G, Eds *Tissue Repair, Contraction and the Myofibroblast*. Springer Science, Chapter 1, pp 8-24

Brown RA and Phillips JB. 2007. 'Cell Responses to Biomimetic Protein Scaffolds used in Tissue Repair and Engineering.' *J Int Review Cytology* 262: 75-150.

Buckingham M. 2003. 'How the community effect orchestrates muscle differentiation.' *BioEssays* 25:13–16

Buffinger N and Stockdale FE. 1994. 'Myogenic specification in somites: induction by axial structures.' *Development* 120: 1443-1452

Burridge K, Fath K, Kelly T, Nuckolls G and Turner C. 1988. 'Focal adhesions: transmembrane junctions between te extracellular matrix and the cytoskeleton.' *Annu Rev Cell Biol* 4:487-525

Burridge K and Chrzanowska-Wodnicka M. 1996. 'Focal adhesions, contractility, and signaling.' *Annu Rev Cell Dev Biol* 12:463–518

Butler-Browne GS, Eriksson PO, Laurent C and Thornell LE. 1988. 'Adult human masseter muscle fibers express myosin isozymes characteristic of development.' *Muscle Nerve* 11:610-620

Cao Y, Vacanti UP, Ma X, Paige KT, Upton J, Chowanski Z, Schloo B, Langer R, Vacanti CA. 1994. 'Generation of neo-tendon using synthetic polymers seeded with tenocytes.' *Transplant Proc* 26:3390-3392

Carmeli E, Moas M, Reznick A and Coleman R. 2004. 'Matrix metalloproteinases and skeletal muscle: a brief review.' *Muscle Nerve* 29: 191–197

Carmeli E, Moas M, Lennon S and Powers SK. 2005. 'High intensity exercise increases expression of matrix metalloproteinases in fast skeletal muscle fibres.' *Exp Physio* 904.:613-619

Chargé SBP and Rudnicki MA. 2004. 'Cellular and molecular regulation of muscle regeneration.' *Physiol Rev* 84:209-238

Charlton CA, Mohler WA and Blau HM. 2000. 'Neural cell adhesion molecule NCAM. and myoblast fusion.' *Dev. Biol.* 221:112-119

Cheema U, Yang SY, Mudera V, Goldspink G and Brown RA. 2003. '3-D in vitro

model of early skeletal muscle development.' *Cell Motil Cytoskeleton* 54: 226-236

Cheema U, Brown R, Mudera V, Yang SY, McGrouther G and Goldspink G. 2004. 'Mechanical signals and IGF-I gene splicing *in vitro* in relation to development of skeletal muscle.' *J Cell Physiol* 9999:1-10

Cheema U. 2004. 'Development and characterisation of 3D skeletal muscle constructs under defined mechanical regulation.' *PhD Thesis*, UCL

Chen CS, Mrksich M, Huang S, Whitesides GM, Ingber DE. 1997. 'Geometric control of cell life and death.' *Science* 276:1425-1428

Chen EH and Olsen EN. 2004. 'Towards a molecular pathway for myoblasts fusion in Drosophila.' *Trends Cell Biol* 14:252-260

Chen L. 1977. 'Alteration in cell surface LETS protein during myogenesis.' *Cell* 10:393-400

Chiquet M, Puri EC, Turner DC. 1979. 'Fibronectin mediates attachment of chicken myoblasts to a gelatine-coated substratum.' *J Biol Chem* 254:5475-5482

Christian CN, Nelson PG, Peacock J and Nirenberg M. 1977. 'Synapse formation between two clonal cell lines.' *Science* 196:995-998

Chromiak J and Vandenburgh HH. 1994. 'Mechanical stimulation of skeletal muscle cell mitigate glucocorticoid-induced decreases in prostaglandin production and prostaglandin synthase activity.'*J Cell Physiol.* 159:407-414

Chromiak JA, Shansky J, Perrone C and Vandenburgh HH. 1998. 'Bioreactor perfusion system for the long-term maintenance of tissue engineered skeletal muscle organoids.' *In Vitro Cell Dev Biol Anim* 34:694-703

Clark E and Brugge J. 1995. 'Integrins and signal transduction pathways: the road taken.' *Science* 268:233-239

Clark P, Coles D and Peckham M. 1997. 'Preferential adhesion to and survival on patterned laminin organizes myogenesis *in vitro.' Exp Cell Res* 230:275-283

Clark P, Dunn GA, Knibbs A, Peckham M. 2002. 'Alignment of myoblasts on ultrafine gratings inhibits fusion in vitro.' *International J biochem and cell biol* 34:816-825

Collins CA, Olsen I, Zammit PS, Heslop L, Petrie Aviva, Partridge TA and Morgan JE. 2005. 'Stem cell function, self-renewal, and behavioural heterogeneity of cells from the adult muscle satellite niche.' *Cell* 122: 289-301

Collinsworth AM, Torgan CE, Nagda SN, Rajalingam RJ, Kraus WE, and Truskey GA. 2000. 'Orientation and length of mammalian skeletal myocytes in response to a unidirectional stretch.' *Cell Tissue Res* 302: 243–251

Corcoran ML, Hewitt RE, Kleiner DE and Stetler-Stevenson WG. 1996. 'MMP-2: expression, activation and inhibition.' *Enzyme Protein* 491-3.:7-19

Cooper ST, Maxwell AL, Kizana E, Ghoddusi M, Hardeman EC, Alexander IE, Allen DG and North KN. 2004. 'C2C12 co-culture on a fibroblast substratum enables sustained survival of contractile, highly differentiated myotubes with peripheral nuclei and adult fast myosin expression.' *Cell motility and the cytoskeleton* 58: 200-211

Cossu G, Kelly R, Di Donna S, Vivarelli E and Buckingham M. 1995. 'Myoblasts differentiation during mammalian somitogenesis is dependent upon a community effect.' *Proc Natl Acad Sci USA* 92:2254-2258

Cronin EM, Thurmond FA, Bassel-Duby R, Williams RS, Wright WE, Nelson KD, GarnerHR. 2004. 'Protein-coated polyL-lactic acid. fibers provide a substrate for differentiation of human skeletal muscle cells.' *J Biomed Mater Res* 69A: 373–381

Cukierman E, Pankov R, Stevens DR and Yamada KM. 2001. 'Taking cell-matrix adhesions to the third dimension.' *Science* 294:1708

d'Albis A, Couteaux R, Janmot C and Roulet A. 1989. 'Specific programs of myosin expression in the postnatal development of rat muscle.' *Eur J Biochem* 183: 583-590

d'Albis A, Chanoine C, Mira JC and Couteaux R. 1990. 'Muscle specific response to thyroid hormone of myosin isoform transitions during postnatal development.' *Eur J Biochem* 193:155-161

Denardi C, Ausoni S, Moretti P, Gorza L, Velleca M, Buckingham M and Schiaffino S. 1993. 'Type 2X-myosin heavy chain is coded by a muscle fiber type-specific and developmentally regulated gene.' *Journal of Cell Biology* 123:823-835

Dennis RG and Kosnik PE. 2000. 'Excitability and isometric contractile properties of mammalian skeletal muscle constructs engineered in vitro.' *In Vitro Cell Dev Biol Anim* 36:327-335

Dennis RG, Kosnik PE, Gilbert ME and Faulkner JA. 2001. 'Excitability and contractility of skeletal muscle engineered from primary cultures and cell lines.' *Am. J. Physiol. Cell. Physiol.*, 280: C288-C295

Dickson G, Peck D, Moore SE, Howard Barton C, Walsh FS. 1990. 'Enhanced myogenesis in NCAM-transfected mouse myoblasts.' *Nature* 344:348-351

DiEdwardo CA, Petrosko P, Acarturk TO, DiMilla PA, LaFramboise WA and Johnson PC. 1999. 'Muscle tissue engineering.' *Clin. Plast. Surg.*, 26: 647-656

Discher DE, Paul Janmey P and Wang Y. 2005. 'Tissue cells feel and respond to the stiffness of their substrate.' *Science* 3105751.:1139 – 1143

Eastwood M, McGrouther G and Brown RA. 1994. 'A culture force monitor for measure of contraction forces generated in human dermal fibroblast cultures: evidence for cell-matrix mechanical signalling.' *Biochim Biophysica Acta* 12012.:186-192

Eastwood M, Porter R, Khan U, McGrouther G and Brown RA. 1996. 'Quantitative analysis of collagen gel contractile forces generated by dermal fibroblasts and the relationship to cell morphology.' *J Cell Physiol* 166:33-42

Eastwood M, Mudera V, McGrouther G and Brown R. 1998. 'Effect of precise mechanical loading on fibroblast populated collagen lattices: morphological changes.' *Cell Motil Cytoskeleton* 40:13–21

Eriksson PO. 1982. 'Muscle fibre composition of the human mandibular locomotor system.' *Swed. Dent. J. Suppl.* 12:1-44

Eriksson PO and Thornell LE. 1983. 'Histochemical and morphological muscle-fibre characteristics of the human masseter, the medial pterygoid and the temporal muscles.' *Archs Oral Biol* 28: 781–95.

Faulkner JA and Dennis RG. 2003. 'Excitability and contractility of skeletal muscle: measurements and interpretation.' In: Guilak F, Butler DL, Goldstein SA, Mooney DJ Eds. Functional Tissue Engineering. Springer New York, p 69-79

Florini JR, Ewton EZ, and Magri KA. 1991. 'Hormones, growth factors, and myogenic differentiation.' *Annu Rev Physiol* 53:201-216

Freed LE, Vunjak-Novakovic G, Biron RJ, Eagles DB, Lesnoy DC, Barlow SK and Langer R. 1994. 'Biodegradable polymer scaffolds for tissue engineering.' *Biotechnology* 12:689-693

Freshney RI. 1992. 'Introduction to basic principles.' In: Animal Cell Culture, Second Edition pp. 1-13. Oxford University Press, Oxford.

Ganong WF. 1988. 'Review of medical physiology.' Appleton and Lange, Conneticut, USA

Gelse K, Poschl E and Aigner T. 2003. 'Collagens – structure, function, and biosynthesis.' *Adv Drug Deliv Rev* 55: 1531-1546

Gilbert SF. 2006. 'Developmental Biology; 6th edition.' Sinauer Associates, Inc., USA

Giraud Guille MM, Mosser G, Helary C and Eglin D. 2005. 'Bone matrix like assemblies of collagen: From liquid crystals to gels and biomimetic materials.' *Micron* 36:602-608

Goichberg P and Geiger B. 1998. 'Direct involvement of N-cadherin-mediated signalling in muscle differentiation.' *Mol Biol Cell* 9:3119-3131

Goldspink G, Scutt A, Loughna PT, Wells DJ, Jacnicke T and Gerlach GF. 1992. 'Gene expression in skeletal muscle in response to stretch and force generation.' *Am. J Physiol* 262: R356-R363

Goldspink G. 1998. 'Cellular and molecular aspects of muscle growth, adaptation and ageing.' *Gerodontology* 151.:35-43

Gorza L. 1990. 'Identification of a novel type 2 fiber population in mammalian skeletal muscle by combined use of histochemical myosin ATPase and anti-myosin monoclonal antibodies.' *Journal of Histochemistry and Cytochemistry* 38:257-265

Grande DA, Halberstadt C, Naughton G, Schwartz R, Manji R. 1997. 'Evaluation of matrix scaffolds for tissue engineering of articular cartilage grafts.' *J Biomed Mater Res.* 34: 211–220

Grinnell F. 1994. 'Fibroblasts, myofibroblasts and wound contraction.' *J Cell Biol* 1244:401-404

Grinnell F. 1999. 'Signal transduction pathways activated during fibroblast contraction of collagen matrices.' In: Desmouliere A, Tuchweber B, Eds. *Tissue repair and fibrosis* Springer Press, Berlin, pp. 61-73

Grinnell F. 2003. 'Fibroblast biology in three-dimensional collagen matrices.' *Trends Cell Biol.* 135:264-9

Grounds MD, McGeachie JK, Davies MJ, Sorokin LM, Maley MAL 1998. 'The expression of extracellular matrix during adult skeletal muscle regeneration: how the basement membrane, interstitium and myogenic cells collaborate.' *Basic Appl Myology* 8:129-141

Gu M, Wang W, Song WK, Cooper DNW, Kaufman SJ. 1994. 'Selective modulation of the interaction of α7β1 integrin with fibronectin and laminin by L-14 lectin during

skeletal muscle differentiation.' *J Cell Sci* 107: 175-181

Guérin CW and Holland PC. 1995. 'Synthesis and secretion of matrix-degrading metalloproteases by human skeletal muscle satellite cells.' *Develop Dyn* 202:91-99

Gulati A, Reddi A and Zelewski A. 1983. 'Changes in the basement membrane zone components during skeletal muscle fiber degeneration and regeneration.' *J Cell Biol* 97:957-962

Gurdon JB, Lemaire P and Kato K. 1993. 'Community effects and related phenomena in development.' *Cell* 755.: 831-4

Halevy O, Novitch BG, Spicer DB, Skapek SX, Rhee J, Hannon GJ, Beach D, Lassart AB.1995. 'Correlation of Terminal Cell Cycle Arrest of Skeletal Muscle with Induction of p21 by MyoD.' *Science* 267: 1018-1021

Hasty P, Bradley A, Morris JH, Edmondson, Venuti JM, Olson EN and Klein WH. 1993. 'Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene.' *Nature* 364:501-506

Herman BA and Fernandez SM. 1978. 'Changes in membrane dynamics associated with myogenic cell fusion.' *J Cell Physiol* 94:253-264

Herman BA and Fernandez SM. 1982. 'Dynamics and topographical distribution of glycoproteins during myoblast fusion: a resonance energy transfer study.' *Biochemistry* 21;3275-3283

Hoh JF. 1991. 'Myogenic regulation of mammalian skeletal muscle fibres.' *News Physiol Sci* 6:1-6

Hollenberg SM, Cheng PF and Weintraub H 1993. 'Use of a conditional MyoD transcription factor in studies of MyoD trans-activation and muscle determination.' *Proc Natl Acad Sci USA* 90: 8028-8032

Horton MJ, Brandon CA, Morris TJ, Braun TW, Yaw KM and Sciote JJ. 2001. 'Abundant expression of myosin heavy-chain IIB RNA in a subset of human masseter muscle fibres.' *Archives of Oral Biology* 46: 1039–1050

Hotchin NA and Hall A. 1995. 'The assembly of integrin adhesion complexes requires both extracellular matrix and intracellular rho/rac GTPases.' *J Cell Biol* 131: 1857-1865

Huang S and Ingber DE. 1999. 'The structural and mechanical complexity of cell growth control.' *Nature Cell Biol* 1: E131-E138

Huang HH, Kamm RD, Lee RT. 2004. 'Cell mechanics and mechanotransduction: pathways, probes, and physiology.' *Am J Physiol Cell Physiol* 287: C1–C11

Huang Y-C, Dennis RG, Larkin L, Baar K. 2005. 'Rapid formation of functional muscle in vitro using fibrin gels.'*J Appl Physiol* 98: 706–713

Huxley AF and Niedergerke R. 1954. 'Structural changes in muscle during contraction. Interference microscopy of living muscle fibres.' *Nature* 173:971–973

Huxley HE and Hanson J. 1954. 'Changes in the cross-striations of muscle during contraction and stretch and their structural interpretation.' *Nature* 173:973–976

Illa I, Leon-Monzon M and Dalakas MC. 1992. 'Regenerating and dernervated human muscle fibers and satellite cells express neural cell adhesion moleculer recognized by monoclonal antibodies to natural killer cells.' *Ann Neurol* 31: 46-52

Ingber DE. 1990. 'Fibronectin controls capillary endothelial cell growth by modulating cell shape.' *Proc Natl Acad Sci USA* 87:3579-3583

Ingber DE. 1998. 'In search of cellular control: signal transduction in context.' *J Cell Biochem* 71:S30-31:232-237

Ingber DE. 2002. 'Mechanical signaling, and the cellular response to extracellular matrix in angiogenesis and cardiovascular physiology.' *Circ Res* 91: 877–887

Irintchev A, Rosenblatt JD, Cullen MJ, Zweyer M, Wernig A. 1998. 'Ectopic skeletal muscles derived from myoblasts implaned under the skin.' *J Cell Sci* 111 22.: 3287-3297

Isaeva VV. 1980. 'Contact orientation of myoblasts and muscle fibres in the differentiating culture of myogenic cells.' *Ontogenez* 11: 181-187

John HA and Lawson H. 1980. 'The effect of different collagen types used as substrata on myogenesis in tissue culture.' *Cell Biol Int Rep* 4: 841-850

Juliano RI and Haskil S. 1993. 'Signal transduction from the extracellular matrix.' *J Cell Biol* 120:577-585

Kao WW and Prockop DJ. 1977. 'Proline analogue removes fibroblasts from cultured mixed cell population.' *Nature* 266:63-64

Karamichos D, Brown RA and Mudera V. 2006. 'Complex dependence of substrate stiffness and serum concentration on cell-force generation.' *J Biomed Mater Res* 78A:407–415

Karamichos D. 2006. 'Effect of strain and stiffness on matrix remodelling genes.' *PhD Thesis*, UCL

Karamichos D, Brown RA and Mudera V. 2007. 'Collagen stiffness regulates cellular contraction and matrix remodeling gene expression.' *J Biomed Mater Res* 83A: 887-894

Kaufman SJ and Foster RF. 1985. 'Remodeling of the myoblast membrane accompanies development.' *Devs Biol* 110(1): 1-14

Kaufman SJ and Foster RF. 1988. 'Replicating myoblasts express a muscle-specific phenotype.' *Proc Natl Acad Sci USA* 85: 9606-9610

Kim B-S, Nikolovski J, Bonadio J, Mooney DJ. 1999. 'Cyclical mechanical strain regulates the development of engineered smooth muscle tissue.' *Nat Biotechnol* 17:979-983

Kjær, M. 2004. 'Role of extracellular matrix in adaptation of tendon and skeletal muscle to mechanical loading.' *Physiol Rev* 84: 649-698

Knudsen, KA. 1990. 'Cell adhesion molecules in myogenesis.' *Curr Opinion Cell Biol* 2:902–906

Korfage JAM and Van Eijden TMGJ. 1999. 'Regional differences in fibre type composition in the human temporalis muscle.' *J Anat* 1999. 194:355-362

Korfage JAM and Van Eijden TMGJ. 2003. 'Myosin Heavy-chain Isoform Composition of Human Single Jaw-muscle Fibers.' *J Dent Res* 826:481-485

Korfage JAM, Koolstra JH, Langenbach GEJ and van Eijden TMGJ. 2005a. 'Fiber-type Composition of the Human Jaw Muscles—Part 1.: Origin and Functional Significance of Fiber-type Diversity.' *J Dent Res* 849.:784-783

Korfage JAM, Koolstra JH, Langenbach GEJ and van Eijden TMGJ. 2005b. 'Fiber-type Composition of the Human Jaw Muscles—Part 2.: Role of Hybrid Fibers and Factors Responsible for Inter-individual Variation.' *J Dent Res* 849.:784-793

Kosnik PE, Faulkner JA and Dennis RG. 2001. 'Functional development of engineered skeletal muscle using polymer fiber arrays.' *Tissue Eng.*, 7: 573-584

Kosnik PE, Dennis RG and Vandenburgh HH. 2003. 'Tissue Engineering Skeletal Muscle.' In: Guilak F, Butler DL, Goldstein SA, Mooney DJ Eds. Functional Tissue Engineering. Springer New York, pp 377-392

Langer R and Vacanti JP. 1993. 'Tissue Engineering.' *Science* 14:2605110.:920-926

Larsson L and Moss RL. 1993. 'Maximum velocity of shortening in relation to myosin isoform composition in single fibres from human skeletal muscles.' *J Physiol* 472:595-614

Lawson-Smith MJ and McGeachie JK. 1998. 'The identification of myogenic cells in skeletal muscle, with emphasis on the use of tritiated thymidine autoradiography and desmin antibodies.' *J Anat* 192:161-171

Lazarides E and Hubbard BD. 1976. 'Immunological characterization of the subunit of the 100 A filaments from muscle cells.' *Proc Natl Acad Sci USA* 73: 4344-4348

Lazarides E. 1980. 'Intermediate filaments as mechanical integrators of cellular space.' *Nature* 283: 249-255

Lequerica JL, Mirabet V, Montero JA, Hurtado C, Piquer S and Carbonell F. 1999. '*In vitro* proliferation, differentiation and immuno-magnetic bead purification of human myoblasts.' *Ann Transplant* 2:103-106

Lewis MP, Tippett HL, Sinanan ACM, Morgan MJ and Hunt NP. 2000. 'Gelatinase-B Matrix Metalloproteinase-9; MMP-9. secretion is involved in the migratory phase of human and murine muscle cell cultures.' *J Muscle Res and Cell Mot* 21: 223-233

Lewis MP, Machell JRA, Hunt NP, Sinanan ACM and Tippett HL. 2001. 'The extracellular matrix of muscle – implications for manipulation of the craniofacial musculature.' *Eur J Oral Sci* 109: 209-221

Lewis MP, Mudera V, Cheema U, and Shah R. 2009. 'Muscle Tissue Engineering.' In: Meyer U, Handschel J, Wiesmann HP, Meyer TH, Eds *Fundamentals of Tissue Engineering and Regenerative Medicine* Springer Science (In Press)

Livak KJ and Schmittgen TD. 2001. 'Analysis of relative gene expression data using real-time quanititative PCR and the $2^{-\Delta\Delta Ct}$ method.' *Methods* 25:402-408

Lluri G and Jaworski DM. 2005. 'Regulation of TIMP-2, MT1-MMP, and MMP-2 expression during C2C12 differentiation.' *Muscle Nerve* 32: 492–499, 2005

Lowey S, Waller GS and Trybus KM. 1993. 'Skeletal muscle myosin light chains are essential for physiological speeds of shortening.' *Nature* 365:454-456

Lutz GJ and Lieber RL. 2000. 'Myosin isoforms in anuran skeletal muscle: their influence on contractile properties and *in vivo* muscle function.' *Microsc Res Tech* 50:443-457

Maroto M, Reshef R, Münsterberg AE, Susan Koester S, Goulding M and Andrew B and Lassar AB. 1997. 'Ectopic *Pax-3* activates *MyoD* and *Myf-5* expression in embryonic mesoderm and neural tissue.' *Cell* 89:139–148

Marenzana M, Wilson Jones N, Mudera V, Brown RA. 2006. 'The origins and regulation of tissue tension: Identification of collagen tension-fixation process in vitro.' *Experimental Cell Research* 312: 423 – 433

Mao JJ, Giannobile WV, Helms JA, Hollister SJ, Krebsbach PH, Longaker MT and Shi S. 2006. 'Craniofacial Tissue Engineering by Stem Cells.' *J Dent Res* 8511.:966-979

Marieb EN. 1998. 'Human anatomy and physiology.' 4th Edition, Benjamin Cummings, Addison Wesley Longman, California, USA

Matrisian LM. 1990. 'Metalloproteinases and their inhibitors in matrix remodeling.' *Trends Genet* 6:121-125

Matrisian LM. 1992. 'The matrix-degrading metalloproteinases.' *BioEssays* 14:455-463

Matthews GG. 2001. 'Neurobiology: molecules, cells and systems.' 2nd Edition Blackwell Science Inc., Massachusetts, USA

Mauck RL, Soltz MA, Wang CCB, Wong DD, Chao P-HG, Valhmu WB, Hung CT, Ateshian GA. 2000. 'Functional tissue engineering of articular cartilage through dynamic loading of chondrocyte-seeded agarose gels.' *J Biomech Eng* 122:252-260

Mauro A. 1961. 'Satellite cells of skeletal muscle fibers.' *J Bio-phys Biochem Cytol* 9:493-496

Mayer U. 2003. 'Integrins: redundant or important players in skeletal muscle?' *J Biol Chem* 278:14587-90

Mayne R, Swasdison S, Sanderson RD, and Irwin MH. 1989. 'Extracellular Matrix, Fibroblasts and the Development of Skeletal Muscle.' *Cellular and Molecular Biology of Muscle Development* New York: Liss, 1989, p. 107–116

McKoy G, Leger ME, Bacou F and Goldspink G. 1998. 'Differential expression of myosin heavy chain mRNA and protein isoforms in four functionally diverse rabbit skeletal muscles during pre- and postnatal development.' *Dev Dyn* 211:193–203

McMinn RMH. 1994. 'Last's Anatomy; 9th Edition.' Churchill-Livingstone, London

Melo F, Carey DJ and Brandan E. 1996. 'Extracellular matrix is required for skeletal muscle differentiation but not myogenin expression.' *J. Cell. Biochem.* 62:227-239

Mikos AG, Sarakinos G, Leite SM, Vacanti JP and Langer R. 1993. 'Laminated three-dimensional biodegradable foams for use in tissue engineering.' *Biomaterials* 14:323-330

Monemi M, Eriksson PO, Eriksson A and Thornell LE. 1998. 'Adverse changes in fibre type composition of the human masseter versus biceps brachii muscle during aging.' *J Neuro Sci* 154: 35–48

Mooney DJ, Hansen L, Vacanti J, Langer R, Farmer S, Ingber D. 1992. 'Switching from differentiation to growth in hepatocytes: control by extracellular matrix.' *J Cell Phys* 151:497-505

Mooney DJ and Mikos AG. 1999. 'Growing new organs.' *Sci Am* 280: 60-65

Moss FP and Leblond CP. 1971. 'Satellite cells as a source of myonuclei in the muscles of growing rats.' *Anat Rec* 170:421-436

Mouly V, Edom F, Barbet JP and Butler-Browne GS. 1993. 'Plasticity of human satellite cells.' *Neuromusc Disord* 3:371-377

Mudera VC, Pleass R, Eastwood M, Tarnuzzer R, Schultz G, Khaw P, McGrouther DA and Brown RA. 2000. 'Molecular responses of human dermal fibroblasts to dual cues: Contact guidance and mechanical load.' *Cell Motil Cytoskeleton* 451.:1-9

Mulder MM, Hitchcock RW, and Tresco PA. 1998. 'Skeletal myogenesis on elastomeric substrates: implications for tissue engineering.' *J Biomat Sci Polym Ed* 9:731-748

Nabeshima Y, Hanaoka K, Hayasaka M, Esumi E, Li S, Nonaka I, Nabeshima Y. 1993. 'Myogenin gene disruption results in perinatal lethality because of severe muscle defect.' *Nature* 364: 532-5

Novikova LN, Novikov LN, and Kellerth JO. 2003. 'Biopolymers and biodegradable smart implants for tissue regeneration after spinal cord injury.' *Curr Opin Neurol* 16(6):711-5

Ohkawa Y, Marfell CGA and Imbalzano AN. 2006. 'Skeletal muscle specification by myogenin and Mef2D via the SWI/SNF ATPase Brg1' *The EMBO Journal* 253:490-500

Okano T and Matsuda T. 1997. 'Hybrid muscular tissues: preparation of skeletal muscle cell-incorporated collagen gels.' *Cell Transplant* 6: 109–118

Okano T and Matsuda T. 1998a. 'Muscular tissue engineering: capillary-incorporated hybrid muscular tissues *in vivo* tissue culture.' *Cell Transplant* 7: 435-442

Okano T and Matsuda T. 1998b. 'Tissue engineered skeletal muscle: preparation of highly dense, highly oriented hybrid muscular tissues.' *Cell Transplant* 7: 71-82

Opas M. 1989. 'Expression of the differentiated phenotype by epithelial cells in vitro is regulated by both biochemistry and mechanics of the substratum.' *Dev Biol* 131:281-293

Park YG, Moon JH and Kim J. 2006. 'A comparative study of magnetic-activated cell sorting, cytotoxicity and preplating for the purification of human myoblasts.' *Yonsei Med J* 472.:179-183

Pavlath GK, Thaloor D, Rando TA, Cheong M, English AW and Zheng B. 1998. 'Heterogeneity among muscle precursor cells in adult skeletal muscles with differing regenerative capacities.' *Dev Dyn* 212:495-508

Pattison JS, Folk LC, Richard W. Madsen RW, Childs TE, and Booth FW. 2003. 'Transcriptional profiling identifies extensive downregulation of extracellular matrix gene expression in sarcopenic rat soleus muscle.' *Physiol Genomics* 15: 34–43

Pedrosa–Domello F, Holmgren Y, Lucas CA, Hoh JFY and Thornell LE. 2000. 'Human Extraocular Muscles: Unique Pattern of Myosin Heavy Chain Expression during Myotube Formation.' *Invest Ophthalmol Vis Sci.* 41:1608–1616

Pedrotty DM, Koh J, Davis BH, Taylor DA, Wolf P and Niklason LE. 2005. 'Engineering skeletal myoblasts: roles of three-dimensional culture and electrical stimulation, *Am J Physiol Heart Circ Physiol* 288:1620-1626

Peppas NA and Langer R. 1994. 'New challenges in biomaterials.' *Science* 263:1715-1720

Periasamy M, Gregory P, Martin BJ, Stirewalt WS. 1989. 'Regulation of myosin heavy-chain gene expression during skeletal-muscle hypertrophy.' *Biochem J* 257:691-698

Pette D and Staron RS. 1997. 'Mammalian skeletal muscle fiber type transitions.' *Int Rev Cytol* 170:143–223

Pette D and Staron RS. 2000. 'Myosin isoforms, muscle fiber types, and transitions.'*Microsc Res Tech* 50:500–509

Polla B, D'Antona G, Bottinelli R and Reggiani C. 2004. 'Respiratory muscle fibres: specialisation and plasticity.' *Thorax* 59:808–817

Powell C, Shansky J, Del Tatto M, Forman DE, Hennessey J, Sullivan K, Zielinski BA, Vandenburgh HH. 1999. 'Tissue-engineered human bioartificial muscles expressing a foreign recombinant protein for gene therapy.' *Hum Gene Ther* 10:565-577

Powell CA, Smiley BL, Mills J and Vandenburgh HH. 2002. 'Mechanical stimulation improves tissue engineered human skeletal muscle.' *Am. J. Physiol.*, 282:C1557-C1565

Prajapati RT, Eastwood M, Brown RA. 2000. 'Duration and Orientation of Mechanical Loads Determine Fibroblast Cyto-mechanical Activation: Monitored by Protease Release.', *Wound Rep. Reg.* 8:239-247

Putnam AJ and Mooney DJ. 1996. 'Tissue engineering using synthetic extracellular matrices.' *Nature Medicine* 27.:824-826

Rawls A, Morris JH, Rudnicki MA, Braun T, Arnold HH, Klein WH and Olsen EN. 1995. 'Myogenin's functions do not overlap with those of MyoD or Myf-5 during mouse embryogenesis.' *Dev. Biol.* 172: 37-50

Rebel JM, Boer WI de, Thijssen CD, Vermey M, Zwarthoff EC and Van der Kwast TH. 1994. 'An *in vitro* model of uroethlial regeneration: effects of growth factors and extracellular matrix proteins.' *J. Pathol.* 173: 283-291

Reynolds JJ, Hembry RM and Meikle MC. 1994. 'Connective tissue degradation in health and periodontal disease nad the roles of matrix metalloproteinases and their natural inhibitors.' *Adv Dent Res* 8:312-319

Reynolds JJ and Meikle MC. 1997. 'The functional balance of metalloproteinases and inhibitors in tissue degradation: relevance to oral pathologies.' *J R Coll Surg Edin* 42:154-160

Riboldi SA, Sampaolesi M, Neuenschwanderc P, Cossu G, and Mantero S. 2005. 'Electrospun degradable polyesterurethane membranes: potential scaffolds for skeletal muscle tissue engineering.' *Biomaterials* 26: 4606–4615

Ringqvist M. 1971. 'Histochemical fiber types and fiber sizes in human masticatory muscles.' *Scand J Dent Res* 79:336-368

Ringqvist M. 1974. 'Fiber types in human masticatory muscles.' *Scand J Dent Res* 82:333-355

Ringqvist M, Ringqvist I, Eriksson P0 and Thornell LE. 1982. 'Histochemical fiber type profile in the human masseter muscle.' *J Neurot Sci* 53:273-282

Rong PM, Teillet MA, Ziller C and Le Douarin NM. 1992. 'The neural tube/notochord complex is necessary for vertebral but not limb and body wall striated muscle differentiation.' *Development* 115:657-672

Rosso F, Giordano A, Barbarisi M and Barbarisi A 2004. 'From cell–ECM interactions to tissue engineering.' *J Cell Physiol* 199:174–180

Rowley JA, Madlambayan G, and Mooney DJ. 1999. 'Alginate hydrogels as synthetic extracellular matrix materials.' *Biomaterials* 20:45-53

Rowley JA and Mooney DJ 2002. 'Alginate type and RGD density control myoblast phenotype.' *J Biomed Mater Res* 60: 217–223

Ruwhof C, van Wamel AE, Egas JM, and van der Laarse A. 2000. 'Cyclic stretch induces the release of growth promoting factors from cultured neonatal cardiomyocytes and cardiac fibroblasts.' *Mol Cell Biochem* 208(1-2):89-98

Sartore S, Mascarello F, Rowlerson A, Gorza L, Ausoni S, Vianello M and Schiaffino S. 1987. 'Fibre types in extraocular muscles: a new myosin isoform in the fast fibres.' *J Muscle Res Cell Motil* 8:161-172

Sasoon D, Lyons G, Wright WE, Lin V, Lassar A, Weintraub H and Buckingham M. 1989. 'Expression of two myogenic regulatory factors myogenin and MyoD1 during mouse embryogenesis.' *Nature* 341: 303-7

Sabourin LA and Rudnicki MA. 2000. 'The molecular regulation of myogenesis.' *Clin Genet* 57:16-25

Saxena AK, Marler J, Benvenuto M, Willital GH, Vacanti JP. 1999. 'Skeletal muscle tissue engineering using isolated myoblasts on synthetic biodegradable polymers: preliminary studies.' *Tissue Eng* 56.:525-532

Saxena AK, Willital GH, Vacanti JP. 2001. 'Vascularised three dimensional skeletal muscle tissue-engineering.' *Biomed Mater Eng* 114.:355-370

Schiaffino S and Reggiani C. 1996. 'Molecular diversity of myofibrillar proteins: gene regulation and functional significance.' *Physiol Rev* 76: 371-423

Schuierer MM, Mann CJ, Bildsoe H, Huxley C, Hughes SM. 2005. 'Analyses of the differentiation potential of satellite cells from *myoD*$^{-/-}$, *mdx*, and *PMP22* C22 mice.' *BMC Musculoskelet Disord* 6: 15

Schultz E and McCormick KM. 1994. 'Skeletal muscle satellite cells.' *Rev Physiol Biochem Pharmacol* 123:213-257

Sciote JJ, Rowlerson AM, Hopper C and Hunt NP. 1994. 'Fibre type classification and myosin isoforms in the human masseter muscle.' *J Neurol Sci* 126: 15–24

Sciote JJ and Morris TJ. 2000. 'Skeletal muscle function and fibre types: the relationship between occlusal function and the phenotype of jaw-closing muscles in human.' *J Orthodontics* 27:15-30

Seale P and Rudnicki MA. 2000. 'A New Look at the Origin, Function, and "Stem-Cell" Status of Muscle Satellite Cells.' *Developmental Biology* 218: 115–124

Shah R, Knowles JC, Sinanan AC, Hunt NP and Lewis MP. 2005. 'Craniofacial muscle engineering using a 3-dimensional phosphate glass fibre construct.' *Biomaterials* 26: 1497–1505

Shansky J, Chromiak J, Del Tatto M and Vandenburgh H 1997. 'A simplified method for tissue engineering skeletal muscle organoids *in vitro.' In Virto Cell Dev Biol – Animal* 33:659-661

Simmons CA and Mooney DJ. 2003. 'Regulation of cellular response to mechanical signals by matrix design.' In: Guilak F, Butler DL, Goldstein SA, Mooney DJ Eds. Functional Tissue Engineering. Springer New York, p 377-392

Sinanan AC, Hunt NP and Lewis MP. 2004. 'Human adult craniofacial muscle-derived cells: neural-cell adhesion-molecule NCAM; CD56.-expressing cells appear to contain multipotential stem cells.' *Biotechnol Appl Biochem.* 401.:25-34

Sinanan AC, Buxton P and Lewis MP. 2006. 'Muscling in on stem cells.' *Biol Cell* 984.:203-14

Singh A, Nelson-Moon ZL, Thomas GJ, Hunt NP and Lewis MP. 2000. 'Identification of matrix metalloproteinases and their tissue inhibitors type 1 and 2 in human masseter muscle.' *Archives of Oral Biology* 45:431-440

Smerdu V, Karsch-Mizrachi I, Campione M, Leinwand L and Schiaffino S. 1994. 'Type IIx myosin heavy chain transcripts are expressed in type IIb fibers of human skeletal muscle.' *Am J Physiol* 267:C1723-C1728

Snow MH. 1978. 'An autoradiographic study of satellite cell differentiation into regenerating myotubes following transplantation of muscle in young rats.' *Cell Tissue Res* 186:535-540

Soussi-Yanicostas N, Barbet JP, Laurent-Winter C, Barton P and Butler-Browne GS. 1990. 'Transition of myosin isozymes during development of human masseter muscle. Persistence of developmental isoforms during postnatal development.' *Development* 108:239-249

Sperber GH. 1989. 'Craniofacial embryology.' Butterworths, London

Stål P. 1994. Characterization of human oro-facial and masticatory muscles with respect to fibre types, myosins and capillaries. Morphological, enzyme-histochemical, immuno-histochemical and biochemical investigations.' *Swed Dent J Suppl* 98:1-55

Stål P, Eriksson PO, Eriksson A and Thornell LE. 1987. 'Enzyme–histochemical differences in fibre-type between the human major and minor zygomatic and the first dorsal interosseus muscles.' *Arch Oral Biol* 32: 833–41

Stål P, Eriksson PO, Schiaffino S, Butler-Browne GS, Thornell LE. 1994. 'Differences in myosin composition between human orofacial, masticatory and limb

muscles: enzyme-, immunohisto- and biochemical studies.' *J Muscle Res Cell Motil* 15: 517–34

Stegemann JP, Hong H and Nerem RM. 2005. 'Mechanical, biochemical, and extracellular matrix effects on vascular smooth muscle cell phenotype.' *J Appl Physiol* 98:2321-2327

Stephenson GMM. 1999. 'Hybrid skeletal muscle fibers: a rare or common phenomenon?'*Clin Exper Pharmacol Physiol* 28:692-702

Stetler-Stevenson WG. 2001. The role of matrix metalloproteinases in tumor invasion, metastasis, and angiogenesis.' *Surg Oncol Clin N Am* 10(2):383-392

Stewart JD, Masi TL, Cumming AE, Molnar GM, Wentworth BM, Sampath K, McPherson JM and Yaeger PC 2003. 'Characterization of proliferating human skeletal muscle-derived cells *in vitro*: differential modulation of myoblast markers by TGF-β2' *J Cell Physiol* 196: 70-78

Strohman RC, Bayne E, Spector D, Obinata T, Micou-Eastwood J, and Maniotis A. 1990. Myogenesis and histogenesis of skeletal muscle on flexible membranes *in vitro*.' *In Vitro Cell Dev Biol Anim* 26:201-208

Struelli C. 1999. 'Extracellular matrix remodelling and cellular differentiation' *Curr Opin Cell Biol* 11:634-640

Sullivan VK, Powers SK, Criswell DS, Tumer N, Larochelle JS, and Lowenthal D. 1995. 'Myosin heavy chain composition in young and old rat skeletal muscle: effects of endurance exercise.' *J Appl Physiol* 78(6):2115-20

Swasdison S and Mayne R 1992. 'Formation of highly organized skeletal muscle fibres *in vitro.*' *J Cell Science* 102:643-652

Tajbakhsh S., Rocancourt D, Cossu G and Buckingham M. 1997. 'Redefining the genetic Hierarchies Controlling Skeletal Myogenesis: *Pax-3* and *Myf-5* Act Upstream of *MyoD.*' *Cell* 89:127-138

Talmadge RJ and Roy RR. 1993. 'Electrophoretic separation of rat skeletal muscle myosin heavy-chain isoforms.' *J Appl Physiol* 75:2337–2340

Tamura RN, Oda D, Quaranta V, Plopper G, Lambert R, Glaser S and Jones JC. 1997. 'Coating of titanium alloy with soluble laminin-5 promotes cell attachment and hemidesmosome assembly in gingival epithelial cells: potential application to dental implants.' *J Periodont Res* 32:287-294

Tardy ME and Kastenbauer ER. 1995. 'Head and Neck Surgery; 2nd Edition Vol 1.' Theime Medical Publishers, Inc, New York

Tatsumi R, Sheehan SM, Iwasaki H, Hattori A and Allen RE. 2001. 'Mechanical stretch induces activation of skeletal muscle satellite cells *in vitro.*' *Exp. Cell Res.*, 267:107-114

Tippett HP. 1997. 'Characterisation of satellite cell cultures derived from the human masseter muscle; a potential model for extracellular matrix regulation?' *M.Sc. Thesis*, University of London

Thornell LE, Billeter R, Eriksson PO and Ringqvist M. 1984. 'Heterogeneous distribution of myosin in human masticatory muscle fibres as shown by immunocytochemistry.' *Archs Oral Biol* 29:1–5

Thorrez L, Shansky J, Wang L, Fast L, VandenDriessche T, Chuah M, Mooney D and Vandenburgh H. 2008. 'Growth, differentiation, transplantation and survival of human skeletal myofibers on biodegradable scaffolds.' *Biomaterials* 29:75-84

Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C and Brown RA. 2002. 'Traction, contraction and contracture: the myofibroblasts and the mechano-regulation of connective tissue remodelling.' *Nature Reviews Molec Cell Biol* 3:349-363

Turner DC, Lawton J, Dollenmeier P, Ehrismann R, and Chiquet M. 1983. 'Guidance of myogenic cell migration by orientated deposits of fibronectin.' *Dev Biol* 95:497-504

Turner DC. 1986. 'Cell-cell and cell-matrix interactions in the morphogenesis of skeletal muscle.' *Dev Biol* 3:205-24

Tuxen A, Bakke M and Kenrad B. 1992. 'Histochemical characterization of masseter muscle fibres in a biopsy study of normal young women.' *Archs Oral Biol* 37:889–93

Vandenburgh HH. 1982. 'Dynamic mechanical orientation of skeletal myofibers in vitro.' *Dev Biol* 93: 438–443

Vandenburgh HH. 1983. 'Cell shape and growth regulation in skeletal muscle: Exogenous vs. endogenous factors.' *J. Cell Physiol* 116: 363-371

Vandenburgh HH. 1987a. 'A computerized mechanical cell stimulator for tissue culture: effects on skeletal muscle organogenesis.' *In Vitro Cellular and developmental biology* 247.:609-619

Vandenburgh HH. 1987b. 'Motion into mass: how does tension stimulate muscle growth.' *Med Sci Sports Exerc* 19: S142–S149

Vandenburgh HH, Karlisch P, and Farr L. 1987. 'Maintenance of highly contractile tissue-cultured avian skeletal myotubes in collagen gel.' *In Vitro* 23: 51

Vandenburgh HH, Karlisch P and Farr L. 1988. 'Maintenance of highly contractile tissue-culture avian skeletal myotubes in collagen gel.' *In Vitro Cell Dev Biol* 24:166-174

Vandenburgh HH, Hatfaludy S, Karlisch P, and Shansky J. 1989. Skeletal muscle growth is stimulated by intermittent stretch/relaxation in tissue culture. *Am J Physiol Cell Physiol* 256:C674–C682

Vandenburgh HH, Swasdison S, and Karlisch P. 1991. 'Computer aided mechanogenesis of skeletal muscle organs from single cells in vitro.' *FASEB J* 5: 2860–2867

Vandenburgh HH. 1992. 'Mechanical forces and their second messengers in stimulating cell growth in vitro.' *Am J Physiol* 262:R350-R355

Vandenburgh H, Del Tatto M, Shansky J, LeMaire J, Chang A, Payumo F, Lee P, Goodyear A, and Raven L. 1996. 'Tissue-engineered skeletal muscle organoids for reversible gene therapy.' *Hum Gene Ther* 7: 2195–2200

Vandenburgh HH. 2002. 'Functional assessment and tissue design of skeletal muscle.' *Ann NY Acad Sci* 961:201-202

van der Ven PFM, Schaart G, Jap PHK, Sengers RCA, Stadhouders AM and Ramaekers FCS. 1992. 'Differentiation of human skeletal muscle cells in culture: maturation as indicated by titin and desmin striation.' *Cell Tissue Res* 270:189-198

Van Wachem PB, van Luyn MJ, and da Costa ML. 1996. 'Myoblast seeding in a collagen matrix evaluated *in vitro.' J Biomed Mater Res* 30:353–360

Velleman SG. 1998. 'The role of the extracellular matrix in muscle development.' *Basic Appl Myology* 8:94 Editorial.

Vogel V and Baneyx G. 2003. 'The tissue engineering puzzle: a molecular perspective.' *Annu Rev Biomed Eng* 5:441–63

Wakelam MJ and Pette D. 1982. 'The control of glucose 1,6-bisphosphate by developmental state and hormonal stimulation in cultured muscle tissue.' *Biochem J* 204: 765–769

Wakelam MJ. 1985. 'The fusion of myoblasts.' *Biochem J* 228: 1–12.

Wang N, Butler JP, Ingber DE. 1993. 'Mechanotransduction across the cell surface and through the cytoskeleton.' *Science* 260:1124-1127

Wang YK, Wang YH, Wang CZ, Sung JM, Chiu WT, Lin SH, Chang YH and Tang MJ. 2003. 'Rigidity of collagen fibrils controls collagen gel-induced down-regulation of focal adhesion complex proteins mediated by $\alpha_2\beta_1$ integrin.' *J Biol Chem* 278:1886-1892

Warren SM, Fong KD, Chen CM, Loboa EG, Cowan AM, Lorenz HP and Longaker MT. 2003. 'Tools and Techniques for Craniofacial Tissue Engineering.' *Tissue Engineering* 9:2: 187-200

Webster C, Pavlath GK, Parks DR, Walsh FS and Blau HM. 1988. 'Isolation of human myoblasts with the fluorescence-activated cell sorter.' *Exp Cell Res.* 174;252-256

Weiss A and Leinwand LA. 1996. 'The mammalian myosin heavy chain gene family.' *Annu Rev Cell Dev Biol* 12:417–439

Wells C, Coles D, Entwistle A, Peckham M 1997. 'Myogenic cells express multiple myosin isoforms.' *J Muscle Res and Cell Mot* 18: 501-515

Whalen RG 1985. 'Myosin isoenzymes as molecular markers for muscle physiology.' *J Exp Biol* 115:43-53, 1985.

Wiid IJF, Boyd CD, Bester AJ, and Van Helden PD. 1984. 'Evidence for transcriptional regulation of the myosin heavy chain during myogenesis.' *Nucleid Acids Research* 12:2717-2729

Williams PL, Warwick R, Dyson M, and Bannister BH. 1989. 'Gray.'s anatomy.' 37th Ed. New York: Churchill Livingstone.

Williams DF. 2006. 'To engineer is to create: The link between engineering and regeneration' *TRENDS in Biotech* 24(1):4-8

Woessner JF. Jr. 1991. 'Matrix metalloproteinases and their inhibitors in connective tissue remodeling.' *FASEB J* 58.:2145-2154

Wright WE, Sasoon DA, and Lin VK. 1989. 'Myogenin, a factor regulating myogenesis, has a domain homologous to MyoD.' *Cell* 56: 607-17

Yablonka-Reuveni Z and Nameroff M. 1987. 'Skeletal muscle cell populations; Separation and partial characterization of fibroblast-like cells from embryonic tissue using density centrifugation.' *Histochemistry* 87;27-38

Yaffe D. 1968. 'Retention of differentiation pontentialities during prolonged cultivation of myogenic cells.' *Proc Natl Acad Sci USA* 61: 477-483

Yaffe D. 1971. 'Developmental changes preceding cell fusion during muscle differentiation in vitro.' *Exp Cell Res* 66(1):33-48

Yaffe D and Saxel O. 1977. 'Serial passaging and differentiation of myogenic cells isolated from dysgenic mouse muscle.' *Nature* 270: 725-727

Yang S, Alnaqeeb M, Simpson H and Goldspink G. 1997. 'Changes in muscle fibre type, muscle mass and IGF-I gene expression in rabbit skeletal muscle subjected to stretch.' *J. Anat.* 190: 613-622

Yasuda T, Kondo S, Homma T, and Harris RC. 1996. 'Regulation of extracellular matrix by mechanical loading in rat glomerular mesangial cells.' *J Clin Invest* 98: 1991–2000

Ye Q, Zund G, Jockenhoevel S, Hoerstrup SP, Schoeberlein A, Grunenfelder J and Turina M. 2000. 'Fibrin gel as a three dimensional matrix in cardiovascular tissue engineering.' *Eur J Cardiothorac Surg* 17: 587-591

Yu F, Stål P, Thornell LE and Larsson L. 2003. 'Human single masseter muscle fibers contain unique combinations of myosin and myosin binding protein C isoforms.' *J Muscle Res Cell Mot* 23: 317–326, 2002